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# Making Fish Protein Concentrates By Enzymatic Hydrolysis

## A status report on research and some processes and products studied by NMFS

MALCOLM B. HALE

SEATTLE, WA
November 1972

## National Marine Fisheries Service, Special Scientific Report--Fisheries Series

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## ABSTRACT

**Research into biological methods for fish protein concentrate (FPC) preparation which has been carried out within the National Marine Fisheries Service is summarized. The effects of various processing conditions and commercially available proteolytic enzymes on yields and characteristics of water-soluble fish protein hydrolysates are presented. Soluble FPC prepared from red hake (*Urophycis chuss*) tended to be deficient in either tryptophan or histidine, depending on the pH of hydrolysis. Hydrolysis of raw fish with an alkaline protease of *Bacillus subtilis* at pH 8.5 or above gave the best balance of essential amino acids and a high yield of soluble product. Pancreatin also gave very good results at pH 8.5. The protein efficiency ratio (PER) of a totally soluble FPC prepared from alewife (*Alosa pseudoharengus*) was equivalent to that of casein. Soluble products prepared from hake were equivalent to casein as a wheat supplement but not as a sole source of protein. Process outlines and preliminary cost estimates are presented for the production of two types of fish protein hydrolysates. Possible food uses and the flavor problem are discussed. This report includes a literature survey of fish protein modifications by fermentation and selected chemical hydrolysis methods as well as by enzymatic hydrolysis processes.**

## INTRODUCTION

### Fish Protein Concentrates

Fish protein concentrates (FPC) are products derived from fish by processes in which the fish protein is placed in a more highly concentrated and stable form. As a form of animal protein FPC can supplement vegetable proteins very effectively. Added in low concentrations they can markedly improve the nutritive value of bread and many other common foods by supplying certain essential amino acids in which vegetable proteins are deficient. As a stable powder (or paste) produced from species of fish not normally used as food, they are the least expensive source of animal protein.

FPC may be produced by various processes to give products with different costs and properties, thus different application. Most processing methods can be classified as chemical (solvent extraction) or biological (enzymatic and microbial) procedures. During recent years most efforts have involved the use of solvents, usually isopropyl alcohol, and several pilot plants and a few full-scale industrial plants have been constructed. By solvent extraction FPC can be produced that is a bland, nearly odorless, lightly colored, water-insoluble but highly nutritive powder. FPC prepared by biological procedures are usually more flavorful and may have desirable functional properties. In general the biological procedures have not advanced beyond the laboratory or small pilot plant stage.

## Definition of Biological Procedures

The work described in this publication has been called the "Biological Method" within the FPC program of the National Marine Fisheries Service (NMFS). It involved the use of enzymes (biological catalysts) to convert fish protein into a stable concentrate with desirable properties. The enzymes partially break down the proteins in the fish tissue making them more or less soluble and releasing much of the fat (lipid) so that it can be separated. The enzyme systems employed may be the natural enzymes contained in the fish (autolysis), purified commercial enzyme preparations from various biological sources (e.g., plants, animal organs, or microbial cultures), or the enzymes may be supplied by living cultures of microorganisms.

## Objectives of Program

Development of a biological process is being pursued because (1) the product can have special properties, such as solubility, which make it more suitable for certain applications than solvent extracted FPC, (2) it may be possible to produce an acceptable protein concentrate more cheaply through biological means than through solvent extraction, and (3) a biological method with proper controls would be more suitable for use on shipboard or on a small scale in remote areas.

The primary objective of work carried out at the National Center for Fish Protein Concentrate (NCFPC) has been to develop a process for making a totally water-soluble product which will offer distinct advantages for use in such foods as soups and beverages. Although processing costs have been considered throughout the work, the major concern has been for improvement of the amino acid pattern and nutritive value of the soluble product.

# STATE OF THE ART

## Literature Survey

Most of the references described here are included in a FPC bibliography[1] which was compiled for the National Center for FPC by the Special Bibliographies Section of the Library of Congress (Library of Congress, 1970).

[1] Available from the U.S. Department of Commerce, National Technical Information Center, Springfield, VA 22151. Price $3.00.

**Enzymatic hydrolysis of fish.**—Several processes for the enzymatic hydrolysis of fish have been reported in the technical and patent literature. Some of these processes are intended primarily for animal feed products but could be used to produce FPC for human consumption.

A patent by Keyes and Meinke (1966) describes an enzymatic hydrolysis process in which a proteolytic enzyme, an antibiotic and a chelating agent are added to a whole fish slurry. Processing begins on board ship and the fish are readily pumped to a land-based holding tank for final processing to oil, meal, and solubles fractions.

Enzymes produced by the growth of molds of the genus Aspergillus on wheat bran are used to digest fish in a patent by Jeffreys and Krell (1965). Either a water extract or the whole, comminuted culture may be added and results are claimed to be superior to those obtained with purified commercial enzymes. A short-term digestion may be carried out at 45° to 55°C after which the liquefied fish is drum-dried. The use of clean, eviscerated fish is recommended to obtain a product for human consumption. If yeast and a little sugar are added for fermentation combined with hydrolysis at 34°C, it is claimed that a product free of fish odor and taste is obtained.

Preservation and hydrolysis of fish using pepsin at pH 3 is described in a patent by Hasdenteufel (1968). A product yield of 19.5% is claimed.

The preparation of fish hydrolysates by digestion with papain has been reported by Sen et al. (1962) and by Sripathy et al. (1962). They developed conditions for the preparation of hydrolysates rich in peptones and proteoses for possible use in bacterial culture media. A process description with yield data for the hydrolysis of fish with papain has been published by Sripathy, Sen, and Lahiry (1964).

FPC development efforts in France have been concentrated on enzymatic hydrolysis methods. Professor Ploquin and co-workers at the University of Nantes have developed a fish hydrolysis process which uses papain and a relatively

high temperature and short digestion time. A French fishing cooperative at Nantes is presently employing an enzymatic procedure to convert the better quality fish processing wastes to a soluble product for use in calf-feeding rations.

McBride, Idler, and MacLeod (1961), liquefied herring with proteolytic enzymes as well as by ensilage and acid hydrolysis. Liquefaction by ensilage was shown to be due to the naturally occuring enzymes in the fish and not to bacterial action. The acid-hydrolysed product was dark in color and the freed oil had a high free fatty acid content. The most effective enzyme treatment found was digestion of precooked herring with pepsin at pH 2. Bromelin and Rhozyme B-6 were less effective. The main problem with enzymatically liquefied herring was the formation of a very stable oil-protein emulsion.

A liquefied fish protein (LFP) has been prepared by Higashi and co-workers in Japan for possible use in infant diets. Fish were hydrolysed with a streptomyces protease in the presence of a high boiling solvent such as xylene or toluene. The liquefied product was steam distilled prior to concentration or drying. Studies on the odor and peptide compositions of LFP have been reported by Onishi and Higashi (1968).

Ehlert (1962) has hydrolysed mechanically disintegrated fish using a dead culture of lactic acid bacteria as the source of hydrolytic enzymes. It is claimed that superior oil separation can be obtained and that the hydrolysate, adjusted to below pH 4, is quite stable. The Lumino Feed Company of Denmark, the patent assignee, is presently marketing an animal-feed ingredient produced by this process.

The relative activities of a wide variety of commercially available proteolytic enzymes acting on a fish protein substrate have been reported by Hale (1969). Saha (1940) found that pepsin and trypsin digested undercooked fish more completely than fish protein that was raw, fully cooked, or fried.

A fish protein concentrate has been prepared in Japan by Dr. Tomiyama (1968) by autolysis of sardines under acid conditions. No additional enzymes were used but chloratetracycline was added as a preservative. A breast milk substitute which included 12% autolysate and 52% wheat flour was formulated and used successfully in infant feedings.

Duncan Law and David Crawford of the Oregon State University Seafood Research Laboratory have liquefied fish by pumping the finely comminuted material through a heat exchanger during a short autolysis period. A process whereby the autolysate is prepared and defatted with a hexane-isopropyl alcohol mixture is described in a patent by Lum (1969).

A patent by Bedford (1957) describes the hydrolysis of fish and fish material by reaction with raw tuna viscera. Preliminary hydrolysis at pH 5.5-6 is followed by hydrolysis at pH 8 to make full use of the visceral enzymes. It is claimed that the addition of minor amounts of NaCl and urea increase the digestion rate substantially.

Ensilage of fish, consisting of hydrolysis by native enzymes under sufficiently acid conditions to prevent bacterial growth, has been studied for many years in the Scandinavian countries. Fish silage is produced industrially for animal feeding. Work carried out at the Danish Ministry of Fisheries with herring has been reviewed by Hansen and Schmidtsdorff (1968).[2] A novel sieve plate apparatus was developed for the separation of suspended solids from hydrolysate and oil fractions.

Formic acid is prefered for fish ensilage because it makes the free amino acids less available to microorganisms and the product is stable at a less acid pH. A silage process involving serial additions of HCl, $H_2SO_4$, and formic acid is the subject of a patent by a Polish company, Centralne Laboratorium Przemyslu Rybnego (1962). A method for preparing fish silage on a small scale using a bitumen-coated metal drum has been described in an Indian Fisheries Bulletin (Anonymous, 1964).

Some information is available on the nutritive value of fish protein hydrolysates. Sripathy et al. (1963) studied the influence of degree of hydrolysis on the nutritive value of fish hydrolysates prepared with papain. A 17-hr hydrolysate had a higher nutritive value than did two short-term hydrolysates, but the protein efficiency ratio (PER) was slightly below that of skim-milk powder. The short-term hydrolysates had

[2] Hansen, P., and W. Schmidtsdorff. 1968. Fish protein hydrolysate: A short review of the work carried out in Denmark and suggestions for future research. Presented to the International Association of Fish Meal Manufacturers Annual Conference, Bremen, Sept. 1968.

low tryptophan levels. Apparently tryptophan is released into the soluble phase more slowly than other amino acids during the earlier stages of hydrolysis.

The nutritive values of two samples of "liquefied fish protein" (LFP) were reported by Higashi et al. (1965). Experiments were conducted with jack mackerel, and the nutritive values were determined by rat feeding tests. Sample 1, prepared by digesting the *cooked* material with commercial proteolytic enzyme isolated from *Bacillus subtilis*, showed very low nutritive value and an imbalance of essential amino acids. Sample 2 of LFP, prepared by digesting the *raw* material with a commercial enzyme isolated from streptomyces, showed high nutritive value and its biological value was higher than that of milk casein.

March et al. (1961) determined the nutritive value of "liquid herring" preparations which had been prepared by enzymatic hydrolysis with pepsin and by high-pressure acid treatment (McBride et al., 1961). In chick feeding studies both liquid preparations gave a growth response intermediate between that obtained with herring meal and with condensed herring solubles.

**Acid/alkaline hydrolysis.**—An estimated 30 million pounds of hydrolysed protein are consumed annually in the United States, and nearly all of it is prepared by acid hydrolysis (Connell, 1966). Most is prepared from cheap vegetable sources and is used in relatively small concentrations as a flavoring agent for meat products, soup mixes, snack items, crackers, and other food products. The poor nutritive value of the acid hydrolysates is of little or no concern in most of these food products.

Several processes have been proposed for the acid or alkaline hydrolysis of fish although nutritive value is reduced either through tryptophan destruction by acid or racemization of amino acids by alkali. A process for the acid hydrolysis of whole fish or fish waste under pressure was described in a patent by Ryan and Wilson (1952). Takahashi (1941) has described the hydrolysis of codfish meal with HCl and subsequent treatment of the product.

A mild alkaline hydrolysis was used by Mohanty and Roy (1955) to improve the functional properties of an acid-washed fish protein. An alkaline process has also been used to solubilize solvent extracted FPC (Tannenbaum, Ahern, and Bates, 1970). Use of the solubilized FPC in several food products was also described (Tannenbaum, Bates, and Brodfeld, 1970).

The preservation and digestion of oily fish material in cold aqueous alkali has been described in a patent by Lovern and Hansen (1954). Hydrolysis may be completed at a convenient time by raising the temperature of the digest. A proteinaceous precipitate, removed from the aqueous phase after acidification, may be stabilized by water and acetone washes. The unprecipitated solids may be concentrated by evaporation.

A patent by Libenson and Pirosky (1968) describes the acid precipitation of lipoproteins from an alkali solubilized fish slurry. The "lipid-free" hydrolysate is then deodorized with hydrogen peroxide and neutralized prior to concentration.

**Fish fermentation.**—Modification of fish protein through fermentation is a biological method of preservation which has been used for thousands of years. Fermented fish pastes and sauces form a very important part of the diet in South East Asia today. Fermentation procedures vary locally but the more popular processes and products have been described by Amano (1961) and by Van Veen (1965). In some processes salt is added to whole fish as a preservative and the fish are liquefied by autolysis (e.g., nuoc-mam produced in Viet-Nam). Other processes may start with gutted fish, and the production of proteases or acids by growing microbial cultures is of prime importance. Although fermentation does not improve the nutritive value of the protein, the keeping quality of fermented fish is greatly increased and organoleptic characteristics are generally improved (Van Veen and Steinkraus, 1970).

Nuoc-mam traditionally contains 20% NaCl and requires 6 months to a year to ferment. The high salt content limits the amount that may be consumed by individuals and, of course, the long period of fermentation is expensive. Over 40 years ago a patent was issued (Kahn, 1927) for the production of fish sauce in 1 to 5 days using less than 10% salt. Obviously this product did not have the desired flavor as the traditional method is still the one in use.

The microbiology and chemistry of Nam-pla, a fermented fish sauce of Thailand, has been studied by Saisithi et al. (1966). Total viable

bacterial counts decrease steadily during a 12-month fermentation, and after 9 months approximately 70% of the bacterial isolates were halophiles of bacillus types. These bacteria produced volatile acids in a medium containing acid-hydrolysed rockfish. A diethyl-ether-ethanol extract of the fermented hydrolysate contained the typical aroma of fish sauce.

A proteolytic yeast has been isolated by Bertullo in Uruguay and used in fish fermentations. Characteristics of the yeast and some details of its use are contained in a patent (Bertullo and Hettich, 1961). A stable fish hydrolysate with a pleasant aroma is formed by the growing yeast culture which produces proteases, acids, and alcohol. The process has been improved since publication of the 1961 patent. Less sugar is added and the fermentation reaches the final pH of about 4.3 in 18 to 24 hr. Most of the fat is removed by centrifugation and the dried product is 70% water-soluble. Fermentation of a mixture of 50% fish, 30% soya, and 20% alfalfa has yielded a product with a PER exceeding that of the casein control.

Burkholder et al (1968) have studied fish fermentation with pure cultures of microorganisms selected more for their lipolytic activity than for proteolytic activity. Menhaden was fermented with hundreds of different cultures of bacteria, yeast, and molds obtained from various sources. Organoleptic screening of the fermentation products yielded seven yeasts and one mold for further study. It was found that the mold and three of the yeasts could utilize menhaden tryglycerides as a carbon source. Two of the microorganisms, through utilization of nonprotein nitrogen and triglycerides, were able to reduce the fat content and increase the protein content of a menhaden ferment substantially while producing neutral to pleasant flavors and aromas.

A number of different types of microorganisms have been used to preserve fish and neutralize fish odor and flavor. The use of *Aerobacter aerogenes, Streptococcus lactis,* and other carbohydrate fermenting microorganisms with fish to which sugar or molasses has been added is the subject of a Japanese patent (Tada and Nakayama, 1956). The keeping quality of the fish is greatly improved by fermentation.

Krishnaswamy, Kadol, and Revankar (1965) fermented a freshwater fish with a pure culture of *Streptococcus lactis* using lactose as the carbon source. The dried product was cream colored with a protein content of about 72%. Levels of total and available lysine, tryptophan, and sulfur-containing amino acids were very good. The biological value of the product as determined by PER, NPU (net protein utilization), and net protein ratio was not significantly different from that of skim-milk powder.

## Summary of NMFS Work (Generalized)

**In-house.**—In-house research within the NMFS has been oriented toward development of a totally water-soluble FPC through the use of enzymatic hydrolysis (Fig. 1). The basic process outline includes enzymatic digestion of a whole fish slurry with control of pH and temperature, screening out of bones and scales, and separation of undigested solids by centrifugation. The clarified hydrolysate is spray-dried to yield a soluble product consisting of peptides, polypeptides, and some free amino acids.

Initial work at College Park included small-scale tests in laboratory flasks with the enzymes pepsin, trypsin, bromelin, ficin, and papain. With the installation of a 30-gal fermentation vessel (Fig. 2) complete with pH and temperature recording and control, larger batches were hydrolysed and soluble products were dried on a small vacuum drum dryer. A light yellow product with a pleasant cheeselike aroma and taste was prepared by hydrolysis of red hake (*Urophycis chuss*) with Rhozyme P-11 at pH 8.5. Digestion of presterilized hake yielded a whiter, milder flavored product at the expense of soluble product yield.

Several large hydrolysate batches were prepared for use in trials of equipment proposed for use in a biological process model scale unit. Centrifugation and spray-drying equipment were tested at the plants of several manufacturers and tests of other components of the model unit were performed at the College Park laboratory. Contractor for design of the system was Artisan Industries of Waltham, Mass.

*Bertullo process.*—For a 2-month period the laboratory processing equipment was used in cooperation with Dr. Victor Bertullo of the University of Uruguay, Montevideo. He had

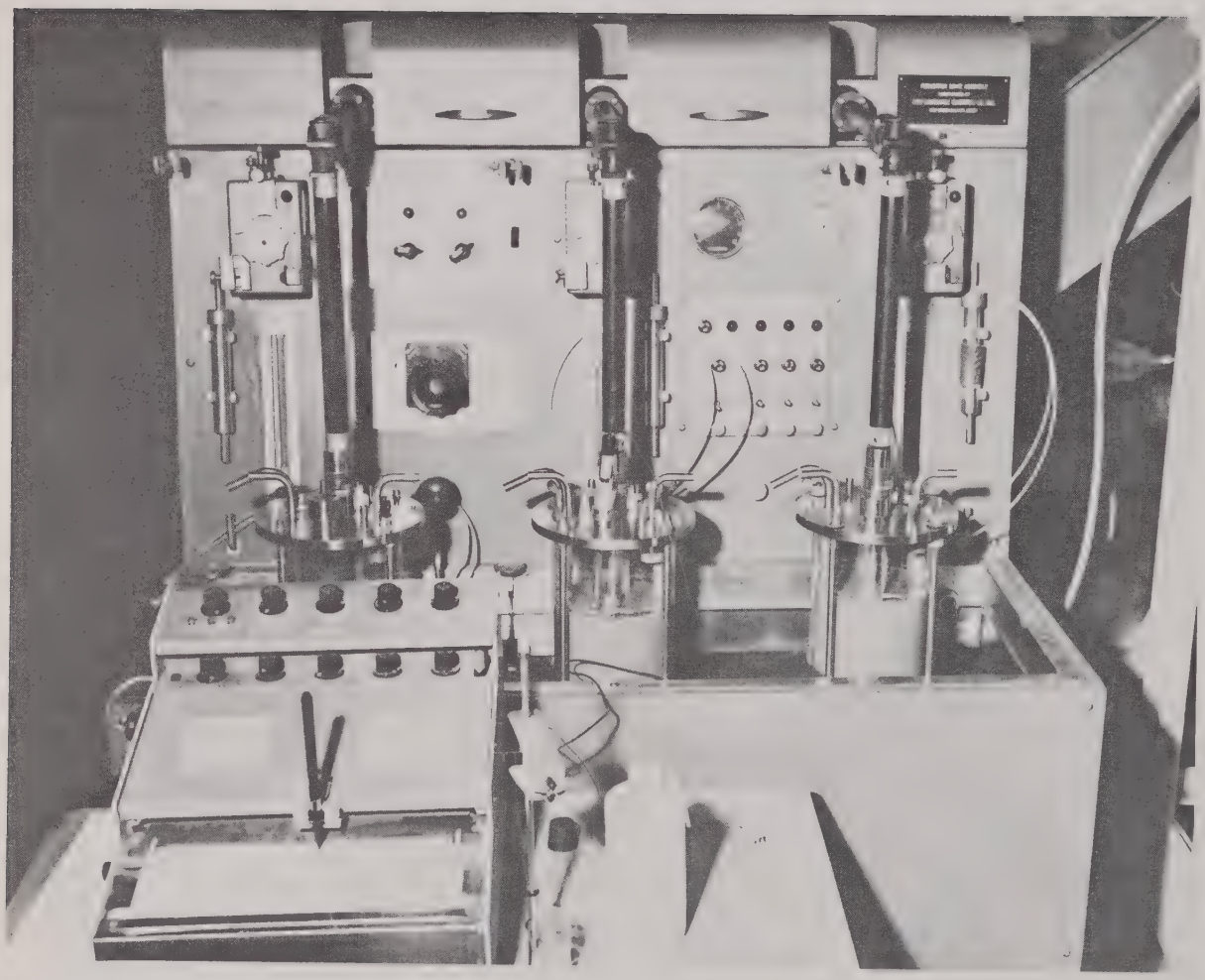

Figure 1.—Experimental system used for fish protein hydrolysis in 5-liter batches.

isolated a proteolytic yeast which is used to hydrolyse and preserve fish in a fermentation process. Batches of red hake, whiting (*Merluccius bilinearis*), and mullet (*Mugil cephalus*) were successfully processed in the laboratory. The drum-dried, whole slurry products were rather dark and hygroscopic. Feeding studies indicated that the products were poor as a sole source of protein but were valuable in a supplementary role. Process improvements have since been made by Dr. Bertullo.

*Relative activities of commercial enzymes.*—A variety of commercially available proteolytic enzymes were collected and compared for relative activity on a specially prepared fish protein substrate. After cooking and removal of solubles by water extractions, muscle tissue of haddock (*Melanogrammus aeglefinus*) was freeze-dried and extracted with ether to yield a powder which was 98% protein on a moisture-free basis. The hydrolysis conditions employed and the relative proteolytic activities which were measured for the various enzyme preparations are described in the section on Summary of Experimental Results. This phase of work was described in a publication by Hale (1969).

*Whole hake products.*—Although tests with the fish muscle substrate showed very large differences in the relative activities of different proteolytic enzymes, differences were much

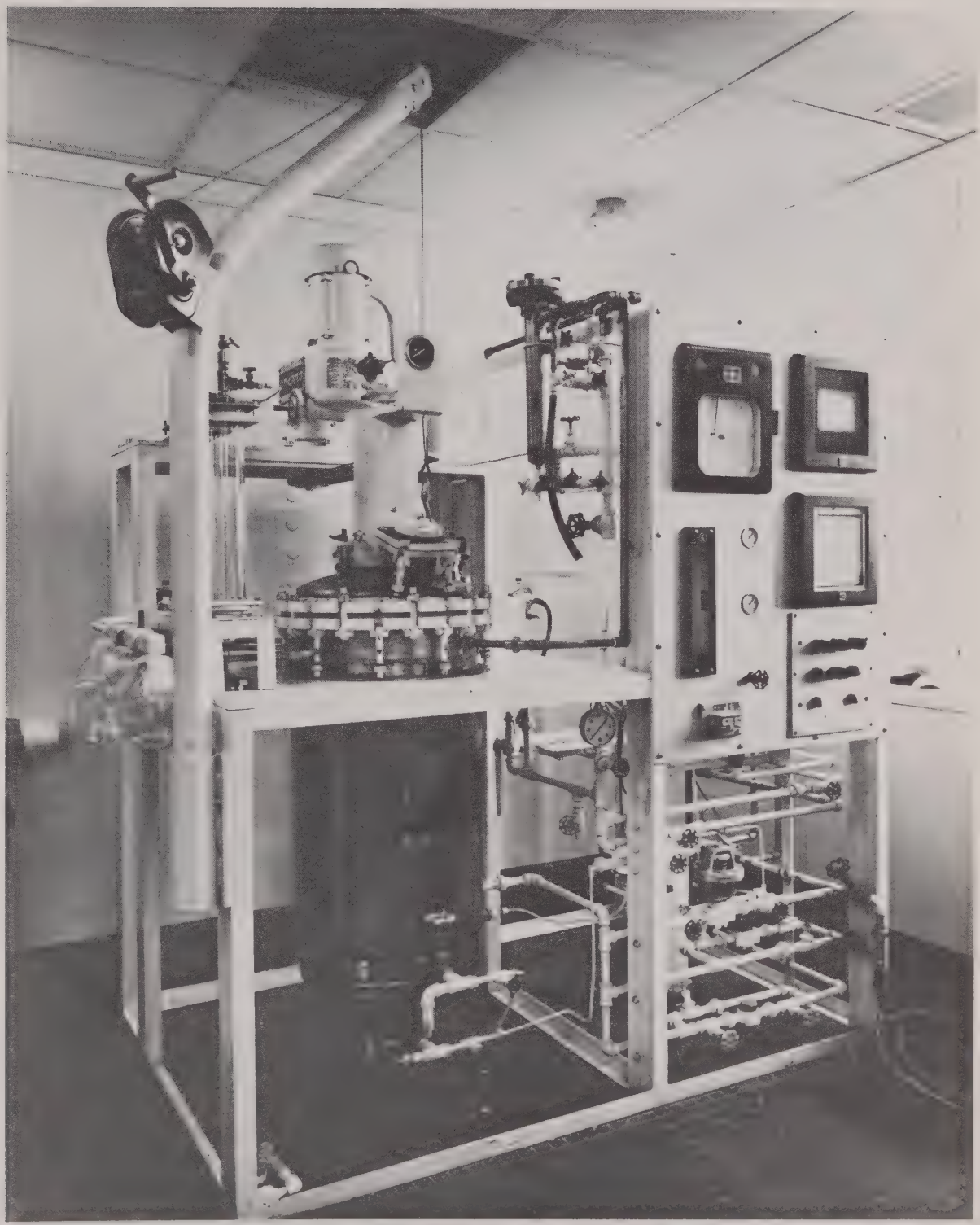

Figure 2.—Fermentor used to prepare FPC in 20-gal hydrolysis batches.

smaller when the enzymes were combined with native fish enzymes in subsequent digestions of raw whole hake. The microbial enzymes Rhozyme P-11 and Bacterial Protease Novo (BPN) gave good yields with whole fish despite relatively low activities as measured by the standard test procedure. Pepsin, which had high relative activity in tests with the standard substrate, gave relatively poor results with the whole raw hake because the native fish enzymes are inactive at pH 2 which is optimum for pepsin.

Limited feeding trials with some early batches of enzymatically hydrolysed hake gave generally poor results. Two batches prepared from raw hake with Rhozyme P-11 and Rhozyme 41 yielded fairly good protein efficiency ratios (PER), but feed consumption and growth rates were low. Subsequent trials of three batches prepared from presterilized hake gave very poor results. Tryptophan and phenylalanine concentrations in the precooked samples were low and tyrosine was absent. Raw hake hydrolysates contained higher concentrations of tryptophan and the phenylalanine-tyrosine combination, but histidine contents were lower.

Feeding trials were suspended while a series of smaller (4- to 5-liter) batches were prepared. A dozen different enzyme preparations, including several newly acquired ones, were employed over a wide range of processing conditions. Amino acid analyses of the soluble products were collected and studied in order to estimate the nutritive quality of various products obtained. During the course of these runs, the spray dryer contained in the model scale installation was put into operation on a regular basis (Fig. 3). The spray-dried products were definitely lighter in color and a little less hygroscopic, on average, than the vacuum drum dried products.

*Amino acid contents.*—Most of the soluble products which were prepared had amino acid analyses which were critically low in either tryptophan or histidine. Tryptophan recovery was very poor at pH 2 (pepsin) but improved with higher pH of hydrolysis and was essentially complete at pH 8. Conversely, histidine recovery was good under acid conditions but very poor at pH 8. These correlations between pH and amino acid concentrations are presented in the experimental results section.

In an attempt to recover adequate concentrations of both tryptophan and histidine in a soluble product, several 2-stage digestions were carried out. After an initial digestion at acid pH, the soluble phase was removed and the undigested solids were treated with a second enzyme at slightly alkaline pH in an attempt to extract more tryptophan. Supernates from the two stages were blended prior to spray drying. Histidine recovery was improved but tryptophan recovery and total yield were poor. The inadequate degree of solubilization in the second stage was probably due to removal of native fish enzymes in the first stage supernate.

*Alkaline bacterial proteases.*—Alkaline proteases of *Bacillus subtilis,* not available for the original testing program, yielded soluble hydrolysates with a better balance of essential amino acids than had been obtained with other enzymes. A series of soluble products were prepared using the experimental enzymes E56 and P58 from Rohm and Haas and also ABP (Alkaline Bacterial Protease) and Alcalase[3] from Enzyme Development Corporation. These enzymes have been developed for use in laundry detergents because of their activity at high pH and temperature. Large-scale use in detergents will probably lead to further reductions in the cost of these enzymes, which is already quite competitive.

Digestion of hake at pH 9 and above with the alkaline proteases gave a better recovery of histidine than had been obtained at pH 7-8.5 with other enzymes. In initial runs at pH 10 and 60°C yields and tryptophan contents were poor but they were much improved by operation at pH 8.5-9.5 and 55°C. Most of the products prepared with alkaline enzymes had fairly good recovery of both histidine and tryptophan, and the probable limiting amino acid became isoleucine.

*FPC solubilizations.*—Several soluble products were prepared by enzymatic hydrolysis of solvent extracted FPC. Pancreatin and ficin as well as the alkaline enzymes were employed. Amino acid patterns of these soluble products were in general superior to those of products prepared from whole hake. Taste and odor problems were

[3] Manufactured by NOVO Industries, Denmark.

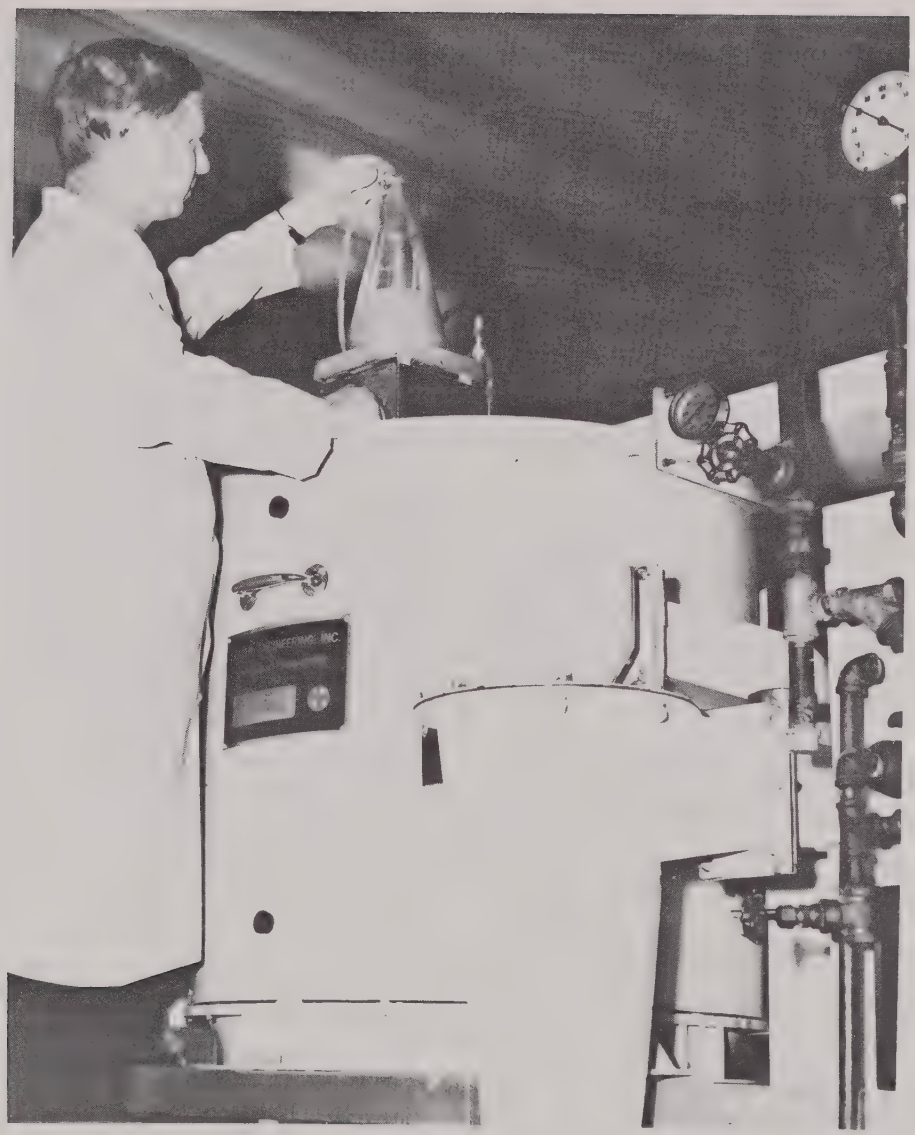

Figure 3.—Spray drying a partially soluble hydrolysate.

minimized with an FPC starting material. Such a process might be most suitable for the preparation of a soluble protein product which would command a high price for use in carbonated beverages.

*Replicate runs with selected enzymes.*—Analysis of the various runs with red hake showed that the hydrolysates prepared with alkaline bacterial proteases had amino acid analyses which ranked highest in terms of chemical scores. Pancreatin hydrolysates ranked second by this method for estimating nutritive value. Both the alkaline enzymes and pancreatin gave average yields of soluble product from fish of about 14%. Average yields with the other enzymes used were lower. Alcalase (an alkaline *B. subtilis* protease, pancreatin, and straight autolysis were chosen for evaluation by replicate runs with both hake and a fatty fish.

Four runs with each of three processes were made with hake. Three runs with each of three processes were made with alewife (*Alosa pseudoharengus*), a fatty fish. Alewife press cake

was prepared and hydrolysed in one of these runs. The press cake hydrolysate and the autolysates of raw fish were partially soluble, whole slurry products which had relatively high fat contents but could be produced at low cost. Material balances, proximate and amino acid analyses, and determinations of PER were obtained for each process.

The hake hydrolysates were equivalent to casein as a supplement to wheat flour protein but inferior as a sole source of protein. By hydrolysis of alewife a totally water-soluble product was finally obtained which was statistically equivalent to casein as a sole source of protein.

## NMFS Contract Research.

*Columbia contract: fish fermentation.*—Under NMFS contracts with the Marine Biology Division of Lamont-Doherty Geological Observatory, Columbia University, investigations into the direct use of microorganisms in the biological treatment and preservation of fish were made.

Screening of microorganisms.—Over 600 different microorganisms were screened for suitability for fish fermentation. These included microorganisms from the culture collection at Lamont Observatory and from major culture collections (e.g., American Type Culture Collection). Many others were isolated especially for the studies from cheeses and other fermented foods, from fish and fish products, and from fish processing equipment.

The isolated microorganisms were used in pure culture fermentations with a lyophilized powder, prepared from whole menhaden, as the substrate. The various cultures were evaluated in terms of lipolytic activity (hydrolysis of menhaden oil), proteolytic activity (hydrolysis of gelatin and of casein), indole and ammonia production (negative factors), and also by subjective organoleptic tests (aroma).

A majority of the microorganisms that were screened produced aromas that were unpleasant to varying degrees. Eight yeasts and molds were selected for further investigation, on the basis of organoleptic characteristics primarily. A fungus, *Geotrichum candidum,* and a yeast, *Candida lipolytica,* were determined to be most promising for menhaden fermentation. Both microorganisms utilized the lipids and nonprotein nitrogen contained in menhaden and effected a reduction in fat content and a net increase in protein content of the ferment while yielding a product with a neutral to pleasant aroma. These results have been published (Burkholder et al., 1968).

Additional studies.—Another strain of *G. candidum,* No. PSM-179, was later isolated from a Peruvian fish meal plant and found to have exceptional lipolytic activity. The two strains of *G. candidum* plus *C. lipolytica* were studied in more detail. The effects of aeration, temperature, initial pH, and time on the growth and lipase production of the cultures were determined. Maximal lipase production and growth of *G. candidum* was achieved after 4 days with mild agitation in a 0.15M phosphate-buffered medium at pH 7.1 and 28°C. With *C. lipolytica,* maximal lipase production was obtained in 24 hr and declined with additional fermentation.

The very active lipase of the PSM-179 strain of *G. candidum* was studied in detail. Determination of the subcellular distribution of the lipase showed that most of the lipolytic activity was associated with the culture medium, indicating that the enzyme is extracellular.

The incorporation of radioactively labeled triglyceride from the substrate by PSM-179 was also studied. Using labeled triolein as substrate, it was found that this strain of *G. candidum* utilized both oleic acid and glycerol as carbon sources. Both of the products of lipolysis were incorporated into the cellular material but a higher percentage of the glycerol was utilized.

Proteolytic microorganisms.—The use of proteolytic microorganisms to produce a nutritious, water-soluble FPC was also studied. A total of 82 new proteolytic microorganisms were obtained from various culture collections and screened to determine relative proteolytic activities. Part of these plus microorganisms from earlier screening tests were selected for further proteolytic fermentation screening experiments. Frozen menhaden, rather than a lyophilized powder, was the substrate used.

One strain of *C. lipolytica* plus a bacterium and a yeast (unidentified) were selected for further studies on the basis of soluble solids and protein determinations. Also two microorgan-

isms with known proteolytic characteristics were further studied: *Bacillus subtilis* (18 strains) and *Rhizopus oligosporus* (4 strains) from various culture collections.

Two-stage fermentations were developed for *B. subtilis* and *R. oligosporus*. The initial stages, at moderate pH for optimum cell growth, were followed by (a) fermentation at pH 10 for optimum activity of the alkaline protease of *B. subtilis* or (b) fermentation of pH 3 for optimum activity of the acid protease of *R. oligosporus*.

Samples of product were prepared for nutritional evaluation at the NCFPC using five selected strains of microorganisms. Menhaden was fermented in a 26-liter fermenter under optimal conditions. Most of the samples had low protein concentrations and high ash contents resulting from pH adjustments. Three of the samples had lipid contents below 1%. In general yields of soluble product and protein recoveries were poor and the direct use of microorganisms for production of totally soluble products does not appear to be an economic practicality.

Studies on lipid stabilization were restricted to developing a package and tray fermentation method of co-fermentation of fish with tempeh or the tempeh fungus *R. oligosporus*. The results were not very promising. Cakes composed of fish-soybean mixtures were not porous as are the soybean cakes used in the normal tempeh fermentation, and difficulties in maintaining moisture controls and the degree of aeration were encountered.

*MIT contract: solubilization of FPC.*—Personnel of the Department of Nutrition and Food Science, Massachusetts Institute of Technology, have, under contracts with the National Marine Fisheries Service, investigated methods for the solubilization of solvent-extracted fish protein concentrate (FPC). An alkaline solubilization method and the use of proteolytic enzymes in both batch and continuous processes have been studied. The objective was to make FPC more suitable for use in food products such as protein beverages.

Alkaline solubilization.—An alkaline process in which FPC was solubilized by a 20-min reaction at pH 12.5 and 95°C has recently been reported by Tannenbaum, Ahern, and Bates (1970). The degree of proteolysis was limited by the short time period, but extensive racemization of constituent amino acids did take place and probably had a detrimental effect on the nutritive value of the product.

The use of the alkaline-process product in several food products was the subject of a second paper (Tannenbaum, Bates, and Brodfeld, 1970). It could be used without further treatment in a number of foods, but off-flavor and color reduction were necessary when it was used in protein beverage formulas. This was effected by passing the non-acid precipitable fraction through charcoal followed by recombination of the fractions.

Proteolytic enzymes.—Investigations of proteolytic enzymes for the solubilization of FPC were divided into two phases. The initial phase consisted of batch studies with a number of different enzyme preparations. The information gained in the batch studies was used in the second phase, a study of continuous reactor in which ultrafiltration membranes could be used to allow removal of soluble products of hydrolysis while retaining the enzyme (also soluble) for continuing reaction with added substrate.

Batch studies.—In batch studies the relative activities of various proteolytic enzymes were determined with respect to FPC solubilization. Pronase (a mixture of proteolytic enzymes from *Streptomyces griseus*), pancreatin, and *Bacillus subtilis* protease (Monzyme) were found to be effective in varying degrees. Methods were devised, or adapted, in order to assay proteolytic activity, particularly the stability of the enzyme during the course of FPC proteolysis in order to estimate enzyme degradation and enzyme inhibition. The proteolytic activity of all enzymes decreased during the course of FPC proteolysis. At 34°C and after 8 hr of hydrolysis, Pronase degradation was estimated to be approximately 30%. At the same time Pronase was inhibited by the products of hydrolysis by a factor of 60%. The influence of pH, temperature, substrate concentration, and enzyme concentration on FPC proteolysis by Pronase, pancreatin, Monzyme, and enzyme combinations was studied.

Gel chromatography indicated that after only 20-min hydrolysis at 34°C most of the soluble peptides released from FPC had molecular weights of less than 2,000. Average molecular weights decreased further with longer hydrolysis periods. The molecular weights of most of the enzymes constituting Pronase were estimated to be in the range of 20,000. These results provided a basis for design of a continuous reactor-ultrafiltration membrane system.

Continuous reactor.—The feasibility of using ultrafiltration membranes with a continuous reactor in order to retain and reuse enzymes was evaluated. Characterization of commercially available membranes was performed in terms of their rejection efficiencies for the various enzyme preparations. A polyelectrolyte complex membrane, PM-10 (Amicon Corp., Lexington, Mass.), demonstrated 100% rejection efficiency for Pronase. Three other membranes which were also tested were only partially retentive for Pronase and Monzyme. The PM-10 membrane also showed a 23% rejection efficiency for the soluble FPC products of hydrolysis. A less retentive membrane or further hydrolysis to smaller peptides would be required for extended continuous operation.

Enzymatic solubilization of FPC was tested experimentally in a small continuous reaction system containing an ultrafiltration membrane. A number of problems were encountered. Feeding of a homogeneous slurry of FPC to the reactor at the required low flow rate was a major problem, but this would be less difficult in a larger scale system. A more serious problem lies in the unstable nature of proteolytic enzymes. Pronase was found to have an inherent low thermal stability and/or self-digestion in the continuous reactor.

Monzyme, a mixture of *B. subtilis* neutral and alkaline proteases manufactured by Monsanto, is relatively stable over a wide range of temperature and pH. Operation with Monzyme at pH 8.8 greatly reduced or eliminated problems of microbial contamination which had been encountered in the continuous reactor at lower pH. Since it is a relatively inexpensive protease the most economical system for FPC solubilization may or may not include an ultrafiltration membrane for enzyme recovery.

The kinetics of FPC solubilization with Monzyme and with pancreatin have been studied in detail and a continuous reactor system which will provide adequate product for nutritional and other evaluations has been assembled. The batch studies on enzymatic solubilization have been described in a publication by Cheftel et al. (1971), and the continuous system studied will be the subject of a later paper.

## Summary of Experimental Results

**Summary of screening tests.**—The results of the enzyme screening tests are listed in Tables 1 and 2. Initial tests were at 40°C and pH 7 for a 1-hr period. Slurries containing 5% by weight of the extracted fish muscle substrate were hydrolysed with several concentrations of each enzyme and both the remaining insoluble solids (washed and dried) and the dissolved solids in the filtrates were measured. A logarithmic plot of a digestion ratio, DR = ratio of solubilized solids to insoluble solids remaining, versus concentration of enzyme gave a straight line over the range of digestion studied. For the 1-hr tests the proteolytic enzymes were compared at DR = 0.5 corresponding to solubilization of one-third of the initially insoluble solids. The relative activities of enzymes tested by the 1-hr hydrolysis are listed in Table 1. Ficin has highest relative activity under these digestion conditions.

The results of 24-hr hydrolysis tests are listed in Table 2. These tests were carried out in 0.2M phosphate buffer at pH 6, 7, or 7.5 and a temperature of 40°, 50°, or 65°C, depending on the optimum activity ranges for the individual enzymes. Sodium benzoate, 0.7%, was included as a preservative. The enzymes are compared at DR = 1.5, corresponding to a solubilization of 60% of the initially insoluble solids.

The more active enzymes as determined by the 24-hr hydrolysis are listed in Table 3 with approximate costs in terms of dollars per pound of bulk enzyme and cents per unit of relative activity as measured. Pancreatin, pepsin, and papain are relatively economical. In later studies with raw whole hake, autolysis played a major role and differences between added enzymes were less pronounced. The effectiveness of pepsin relative to other enzymes is lower with whole hake because autolytic activity is reduced

Table 1.—Relative activities of proteolytic enzymes from 1-hr hydrolysis at 40°C and neutral pH.[1]

| Enzyme | Manufacturer and/or supplier[2] | Enzyme concentration | Relative activity[3] |
|---|---|---|---|
| | | *Weight %* | *g/g* |
| Ficin | Miles | 0.11 | 900.0 |
| Ficin | EDC | 0.21 | 470.0 |
| Ficin | NBC | 0.29 | 350.0 |
| Pronase | Calbiochem | 0.29 | 345.0 |
| Pepsin (1:10,000) | NBC | 0.39 | 260.0 |
| Rhozyme P.F. Conc. | Rohm and Haas | 0.53 | 189.0 |
| Bromelin | NBC | 0.86 | 116.0 |
| Papain | Miles | 1.12 | 89.3 |
| Bacterial proteinase | EDC | 1.23 | 81.0 |
| Trypsin (4 × USP) | NBC | 1.45 | 69.0 |
| Panol | EDC | 1.50 | 67.0 |
| HT proteolytic | Miles | 1.50 | 67.0 |
| Papain | Penick | 3.00 | 33.3 |
| Bromeliq | Miles | 4.25 | 23.6 |
| Papain | NBC | 6.10 | 16.4 |
| Rhozyme P-11 | Rohm and Haas | 11.00 | 9.1 |

[1] Pepsin digestions carried out at pH 2. Activity of pepsin is 1:10,000 based on coagulated egg albumen.
[2] Suppliers of enzymes are listed in the Appendix.
[3] Relative activity is equivalent to the weight ratio (g substrate/g enzyme) at which one-third of the substrate is digested in 1 hr.

at pH 2 which is optimum for pepsin. Conversely, the effectiveness of Rhozyme P-11 and other microbial enzymes in combination with the native enzymes of raw hake is much greater than would be expected on the basis of the results listed in Table 2.

**Autolytic activity of red hake.**—Native proteolytic enzymes play a major role in the hydrolysis of raw, whole red hake and as noted above modify somewhat the apparent relative activities of various added enzymes. In an early phase of the biological program, whole fish slurries were cooked prior to digestion as a standard control measure. There was a significant autodigestion during heat up of the slurry before native enzymes were inactivated. It was found, however, that effective solubilization of precooked fish could not be achieved unless an excessive amount of commercial enzyme was added. Soluble solids were obtained from cooked fish in lower yields with low concentrations of tryptophan and phenylalanine and in many cases a complete absence of tyrosine. For this reason, raw fish were chosen as the standard starting material for enzymatic hydrolyses.

Table 2.—Relative activities of enzymes for 24-hr hydrolysis of fish protein at near optimum pH and temperature.

| Enzyme[1] | Manufacture specified optimum range | | Test conditions | | Relative activity |
|---|---|---|---|---|---|
| | pH | Temp. | pH | Temp. | |
| | | °C | | °C | *g/g* |
| Pronase | 7-8 | 50 | 7.5 | 50 | 1,110 |
| Pepsin (Cudahy, 1:10,000) | 2 | 40-50 | 2.0 | 50 | 1,000 |
| Pepsin (NBC, 1:10,000) | 2 | 40-50 | 2.0 | 50 | 770 |
| Papain (Miles) | 6-8 | 60-70 | 7.0 | 65 | 370 |
| Panol (EDC) | 5-7 | 50-70 | 6.0 | 65 | 294 |
| Papain (Wallerstein) | 5-6 | 60-70 | 6.0 | 65 | 294 |
| Pepsin (Wilson, 1:3,000) | 2 | 40-50 | 2.0 | 50 | 286 |
| Trypsin (Wilson, 1:80) | 7-9 | 40 | 7.5 | 40 | 222 |
| Pancreatin | 7-9 | 40 | 7.5 | 40 | 222 |
| Ficin (Miles) | 5-8 | 30-50 | 6.0 | 40 | 200 |
| Ficin (EDC) | 4-9 | 30-50 | 6.0 | 40 | 167 |
| Trypsin (NBC, 1:80) | 7-9 | 40 | 7.5 | 40 | 145 |
| Rhozyme PF Conc. (Rohm and Hass, 58.92X) | 5-8.5 | 40-60 | 7.0 | 50 | 110 |
| Bromelin (NBC) | 4-9 | 30-60 | 6.0 | 50 | 100 |
| Bacterial Protease Novo | 7 | -- | 7.0 | 50 | 78 |
| HT proteolytic | 6-9 | 50 | 7.5 | 50 | 32.3 |
| Rhozyme P-11 | 6-9 | 40-50 | 7.5 | 50 | 18.9 |
| Prolase (Wallerstein) | 3-8 | 40 | 6.0 | 40 | <10 |
| Bromelin (Miles, 1:10) | 4-9 | 30-60 | 6.0 | 50 | <10 |
| Fungal protease (Miles) | 4-7.5 | 30-50 | 6.0 | 40 | <10 |

[1] Suppliers of enzymes are listed in the Appendix.
[2] Relative activity is equivalent to the weight ratio (g substrate/g enzyme) at which 60% of the substrate is solubilized in 24 hr.

Table 3.—Approximate costs of proteolytic enzymes per unit of relative activity as determined by 24-hr hydrolysis of fish protein.

| Enzyme | Source[1] | Approximate cost | Relative activity | Approximate cost |
|---|---|---|---|---|
| | | *$/lb.* | *g/g* | *¢/unit* |
| Pepsin, 1:10,000 .... | Cudahy | 16.00 | 1,000 | 1.6 |
| Pepsin, 1:3,000 ..... | Wilson | 5.15 | 286 | 1.8 |
| Pancreatin, 3XN.F. .. | Cudahy | 3.50 | 222 | 1.6 |
| Trypsin, 1:80 ..... | Wilson | 4.05 | 222 | 1.8 |
| Panol (papain) ..... | EDC | 7.00 | 294 | 2.4 |
| Papain ..... | Miles | 11.00 | 370 | 3.0 |
| Papain ..... | Wallerstein | 11.50 | 294 | 3.9 |
| Ficin ..... | EDC | 11.00 | 167 | 6.6 |
| Ficin ..... | Miles | 13.50 | 200 | 6.8 |
| Pronase ..... | Calbiochem | 450.00 | 1,110 | 40.0 |

[1] Suppliers of enzymes are listed in the Appendix.

*Effect of cooking on degree of digestion.*—A more recent experiment provided quantitative evidence of the effect of cooking on the degree of digestion of hake. Flasks containing equal weights of comminuted hake and distilled water were cooked for 30 min in a 95°C water bath and then reacted for 5 hr with proteolytic enzymes added at concentrations of 0, 0.2, 1.0, and 5.0% (dry weight basis). An equal number of flasks containing raw hake slurries were reacted at the same enzyme levels. In phase 1 of the experiment, pancreatin was used at 45°C without pH adjustment. The pH dropped during hydrolysis from pH 7.2 to a lower value depending on enzyme concentration (pH 6.5 minimum). In the second half of the experiment the added enzyme was Alcalase,[4] and the pH was maintained at 9.5 during the 5-hr digestion at 55°C. The hydrolysates were acidified, centrifuged, and gravity filtered at the end of the 5-hr digestion period. The percent recoveries of dissolved solids, crude protein, and tryptophan in the filtrates, corrected for added enzymes and salts, were plotted versus enzyme concentration for each set of conditions.

In Figure 4 the yields of dissolved solids from raw and cooked hake are plotted versus the concentration of pancreatin added. The percent yield from raw hake autolysis (zero enzyme added) could have been obtained from cooked hake by adding about 0.7% enzyme. The normally used concentration of pancreatin, 0.5%, results in a yield under the test conditions of about 11%.

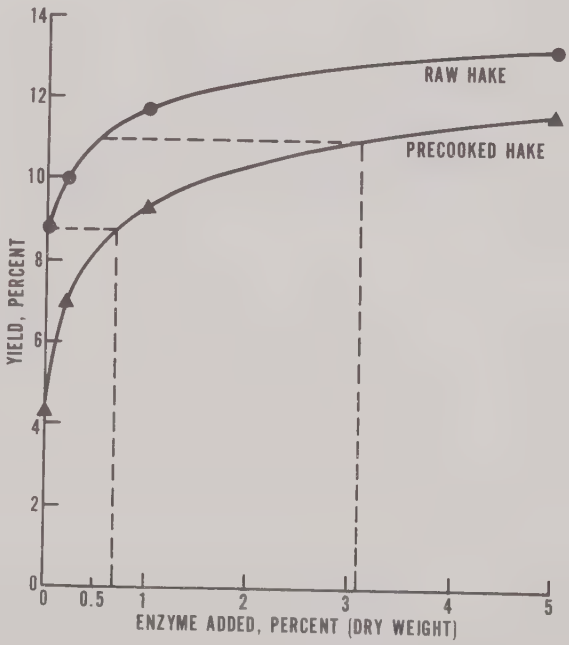


Figure 4.—Yields of soluble solids from raw hake and cooked hake as a function of enzyme concentration. Pancreatin, 5 hr at 45°C, no pH adjustment.

The second dashed line illustrates that a comparable yield from cooked hake would require the addition of about 3.1% enzyme.

In Figure 5 soluble tryptophan recoveries in the filtrates as percent of tryptophan content of the starting material are plotted versus concentration of Alcalase used at pH 9.5. With no enzyme additions similar yields were obtained from raw and cooked hake. A tryptophan recovery from cooked hake equivalent to that from

[4] Alcalase is an alkaline *B. subtilis* protease manufactured by Novo Industries. See Appendix for enzyme listing.

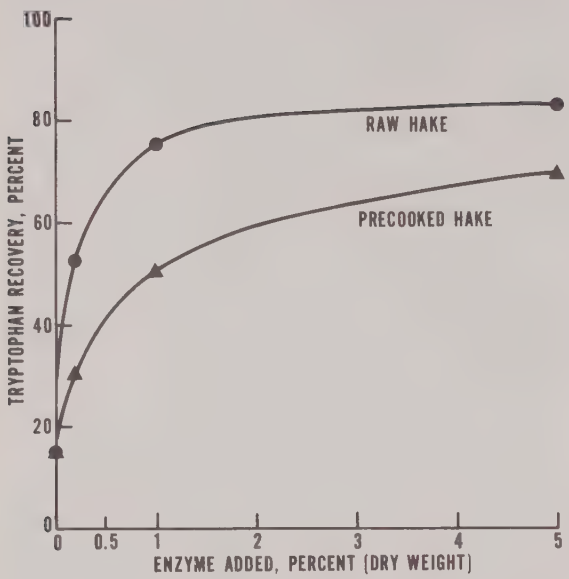


Figure 5.—Soluble tryptophan recovery from raw and cooked hake as a function of enzyme concentration. Alcalase, 5 hr at 55°C and pH 9.5.

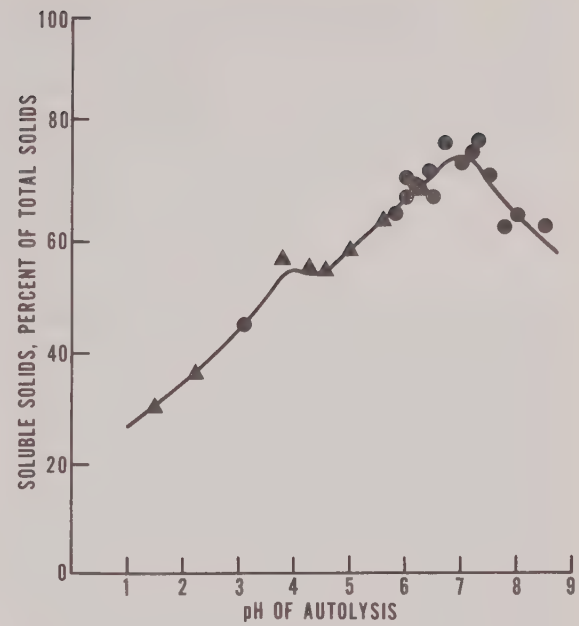


Figure 6.—Percent solubilization versus pH for autolysis of red hake, 24 hr at 50°C.

raw hake with 0.5% Alcalase, however, would require a 7-fold increase in enzyme concentration. Roughly similar results were obtained from plots of crude protein recoveries versus concentrations of added enzymes.

*Autolysis of raw hake.*—The optimum temperature for autolysis of red hake is in the range of 50° to 55°C. This holds even for a 24-hr autolysis although a lower optimum temperature would be expected on the basis of theory. The optimum pH is about 7 as shown in Figure 6 which is based on the 24-hr digestion at 50°C. Approximately optimum conditions are obtained without any pH adjustment in which case the pH drops about one-half unit from an initial value a little above pH 7. Rate of hydrolysis increases with lower substrate concentrations. In one test, a 4-hr autolysis at 55°C, reducing the concentration of fish from 50% of the slurry to 20%, increased degree of solubilization by about 12%. At a concentration of 5% fish solubilization was increased by over 20%. A 50% slurry has been the standard used in our preparation of hydrolysate products, but the optimum concentration must be determined on the basis of several aspects of production economics for a specific process.

Different catches of hake differ in autolytic activity, as would be expected. The addition of commercial enzyme preparations has a greater effect on fish with lower native activity but the total degree of solubilization remains lower if both catches are processed similarly.

*Microbiological aspects.*—Although red hake are not sterilized prior to hydrolysis, it has been definitely established that solubilization is due to native enzymatic activity and not to the action of bacteria. In one experiment hake were eviscerated and the flesh and viscera were ground separately. Flesh-water, viscera-water, and flesh-viscera-water slurries were agitated at 55°C without pH control. Samples were withdrawn at intervals and the amount of solubilized protein was measured. Soluble protein was always least in the flesh-water slurries and greatest in the slurries containing at least 2% viscera. Total bacterial plate counts were less than 300 per gram of raw fish and were essentially zero for all slurries after 24 hr.

A second autolysis experiment involved agitation for 24 hr at 55°C of slurries containing (a) raw hake, (b) cooked hake, (c) raw viscera plus cooked flesh, and (d) cooked viscera plus

Table 4.—Results of autolysis experiments with raw and cooked whole hake and flesh-viscera mixtures.

| Flask # | Contents | Approximate % solubilization | Total bacterial count/gram 24-hr incubation at: | |
|---|---|---|---|---|
| | | | 35°C | 55°C |
| 1 | Raw whole hake | 82.0 | <300 | <300 |
| 2 | Cooked whole hake | 63.5 | $8.8 \times 10^6$ | $1.6 \times 10^7$ |
| 3 | Raw flesh + cooked viscera | 55.1 | <300 | <300 |
| 4 | Cooked flesh + raw viscera | 80.1 | $6.7 \times 10^6$ | $1.07 \times 10^7$ |

raw flesh. Blends of flesh and viscera were in the same proportions as were derived from the beheaded whole fish. The experimental results are presented in Table 4. These results also indicate that autolysis is due to native enzymes, principally in the viscera, rather than to bacterial growth. Both the cooked and raw materials were exposed to atmospheric contamination during transfer to reaction flasks. The high bacterial counts for the cooked hake and the cooked flesh-raw viscera mixture agree with previous experience that cooked fish, at least in the case of red hake, are more susceptible to bacterial spoilage than are raw fish.

### Yields, PER, compositions for various enzymes.

*Yields.*—Average percent yields of soluble solids are listed in Table 5 for groupings of the principal types of proteolytic enzymes employed to hydrolyse red hake. They range from 10% for autolysis without added enzymes to about 14% when an alkaline protease or pancreatin was used. Most of the runs that are included lasted 20 hr or more and employed enzyme concentrations equivalent to a cost of 2 cents per pound of dry solids. Three of the runs with alkaline proteases were at an enzyme cost of 3 cents per pound and only one of the runs with alkaline enzymes lasted over 6 hr.

Table 5.—Average yields of dry solids from red hake solubilized by proteolytic enzymes.

| Enzyme(s) | Number of runs | Yield, dry solubles/wet fish, percent ± standard deviation |
|---|---|---|
| Autolysis | 5 | 10.0 ± 0.4 |
| Pepsin | 1 | 10.9 |
| Papain | 5 | 11.3 ± 0.8 |
| Bacterial Protease Novo | 4 | 11.7 ± 0.10 |
| Bromelin, ficin | 3 | 12.3 ± 0.3 |
| Rhozyme P-11 | 3 | 12.5 ± 0.75 |
| Pancreatin | 7 | 13.8 ± 1.3 |
| Alkaline proteases | 5 | 14.3 ± 1.0 |

*Amino acid compositions.*—Low PER in feeding trials with fish protein hydrolysates could be largely attributed to an imbalance of essential amino acids in the soluble solids fraction. The concept of chemical score was studied as a means for estimating product nutritive quality. The chemical score concept is that nutritive value of a protein is directly related to the concentration of the most limiting amino acid. This amino acid is assumed to be that with the lowest concentration relative to a "perfect" reference protein. Chemical scores were calculated for soluble products on the basis of the most commonly used reference protein, whole egg, and also on the basis of the diet experimentally determined by Ramo Rao, Metta, and Johnson (1959) to give maximum growth of rats. Average chemical scores calculated for soluble products and based on the Rao diet are listed in Table 6 for groupings of similar enzyme type and range of hydrolysis pH. Among the wide range of enzymes used, the limiting amino acids are tryptophan under acid conditions, histidine at slightly alkaline pH, and principally isoleucine with the alkaline proteases at higher pH.

*Tryptophan concentration.*—The concentrations of tryptophan measured in soluble hydrolysates are plotted versus the pH of hydrolysis in Figure 7. A positive correlation is apparent and the tryptophan concentration at pH 8 and above is about 3 times greater than it is in soluble products prepared near pH 2.

Material balances showed that although a little tryptophan may have been destroyed at the lower pH values, the primary problem was one

of distribution. A low tryptophan concentration in the soluble fraction was accompanied by an abnormally high concentration in the insoluble solids remaining. Increasing the yield of soluble product resulted in a higher concentration of tryptophan in the product. This is illustrated in Figure 8. Tryptophan concentration climbs sharply with yield and is at a maximum at soluble solids yields of 13% and above. Since yields from straight autolysis of raw red hake averaged only 10%, it has not been possible to obtain a totally soluble product with adequate tryptophan without the addition of commercial enzymes.

Table 6.—Means and standard deviations of chemical scores for soluble hake products grouped according to type of enzyme and pH of digestion.

| Enzymes | pH range | Number of samples | Chemical score, average ± standard deviation[1] |
|---|---|---|---|
| Pepsin | 2-2.5 | 4 | 25.5 ± 6.9 |
| Autolysis | 4.5 | 3 | 20.7 ± 3.1 |
| Autolysis | 6.6-7 | 4 | 41.3 ± 5.3 |
| Papain, Bromelin, Ficin | 5.7-6.8 | 10 | 42.7 ± 9.3 |
| Bacterial Protease Novo, Monzyme | 6-8 | 5 | 54.0 ± 4.9 |
| Rhozyme P-11, Rhozyme 41 | 8-8.4 | 7 | 45.0 ± 13.4 |
| Pancreatin, Trypsin | 7-9 | 15 | 60.0 ± 5.6 |
| Alcalase, ABP, P-58, E-56 | 8.5-10 | 13 | 64.2 ± 5.4 |

[1] Based on rat requirements as determined by Rama Rao, Metta, and Johnson (1959).

*Histidine concentration.*—The concentration of histidine in soluble products was highest for pepsin hydrolyses at pH 2 and declined with increasing pH of hydrolysis to a minimum recovery in products prepared at pH 8. Fairly good histidine concentrations were obtained in hydrolysates prepared at pH 8.5 and above. Low concentrations of histidine under slightly alkaline conditions are not due to a preferential distribution in the sludge, as is the case with tryptophan. This is illustrated in Table 7 in which amino acid recoveries in the soluble products and in the solubles and sludges combined are listed for four different processes. Digestion at pH 8 with pancreatin resulted in a poor recovery of histidine in both soluble and sludge fractions. A 2-stage digestion, autolysis at pH 4 followed by hydrolysis of the sludge at pH 8, gave a better histidine recovery but tryptophan recovery was

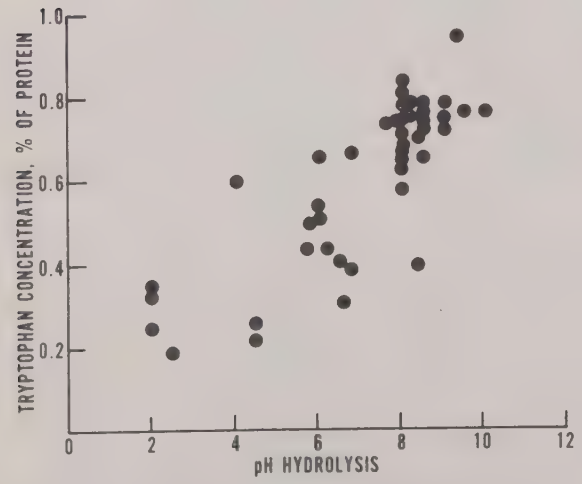


Figure 7.—Effect of pH of hydrolysis on the tryptophan concentration in soluble FPC.

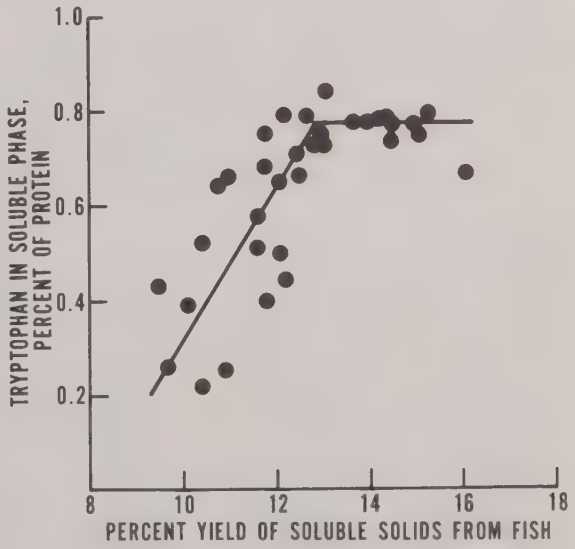


Figure 8.—Tryptophan concentration in soluble solids versus percent yield of solubles from enzymatic hydrolysis of red hake.

poorer. The pepsin digestion at pH 2 involved fish muscle tissue rather than whole hake. Histidine recovery was good but yields of protein and tryptophan in the soluble phase were very poor. Digestion of whole hake at pH 8.5 with the alkaline protease gave good yields of protein and tryptophan combined with a fair recovery of histidine.

*Replicate runs, red hake.*—Replicate runs were carried out with three different processes using red hake as the feed material. Three 6-liter batches and one 20-gal batch were prepared by each process. Digestion conditions used in these runs and similar runs with alewife, a fatty fish, are shown in Table 8. These enzymes and conditions were chosen on the basis of results presented in Tables 5 and 6. Average values of yields, proximate analyses, and essential amino acid concentrations for the raw hake and products resulting from each process are listed in Table 9. The high yield and fat content for autolysis is due to the inclusion of insoluble solids. Two feeding trials were carried out with the products of each process. One was with a blend of the spray-dried products from the three 6-liter batches and the other was from the 20-gal batch.

*Replicate runs, alewife.*—Three 6-liter batches of alewife, a fatty fish, were hydrolysed by each of three processes and blends of the spray-dried products were used in rat feeding trials. Average values of yields, proximate analyses, and essential amino acid concentrations for the raw alewife and products resulting from each process

Table 7.—Recovery of histidine, tryptophan, and other amino acids in soluble product and in solubles plus sludge.

| Run No. | Enzyme | pH | Fraction | Recovery | | | |
|---|---|---|---|---|---|---|---|
| | | | | Protein | Histidine | Tryptophan | Other essential amino acids |
| | | | | % | % | % | % |
| PC-26-1 | Pancreatin | 8 | Soluble product | 72.8 | 42.3 | 68.0 | 69-78 |
| | | | Solubles + sludge | 84.0 | 49.6 | 90.0 | 81-89 |
| AA/PC-26-1 | Autolysis/pancreatin (2-stage) | 4/8 | Solubles | 71.0 | 55.0 | 54.0 | 63-74 |
| | | | Solubles + sludge | 88.0 | 69.4 | 83.0 | 83-90 |
| Pep-Sd-1 | Pepsin | 2 | Solubles | 51.6 | 57.1 | 26.4 | 42-55 |
| | | | Solubles + sludge | 95.0 | 100.0 | 93.0 | 90-100 |
| P58-27-7 | P58 alkaline protease | 8.5 | Solubles | 82.8 | 57.8 | 76.9 | 65-74 |
| | | | Solubles + sludge | 100.6 | 74.3 | 100.0 | 81-93 |

Table 8.—Replicate runs with red hake (*Urophycis chuss*) and alewife (*Alosa pseudoharengus*): products and processing conditions.

| Sample No. | Feed material | Enzyme | Concentration, % of protein | Conditions | | | Product solubility |
|---|---|---|---|---|---|---|---|
| | | | | pH | Temperature | Time | |
| | | | | | °C | hr | |
| II-PC-Blend | Raw hake | Pancreatin | 0.50 | 8.5 | 45 | 5 | Total |
| II-PC-40* | Raw hake | Pancreatin | 0.50 | 8.5 | 45 | 5 | Total |
| II-A-Blend | Raw hake | Alcalase | 0.35 | 9.0 | 55 | 5 | Total |
| II-A-40* | Raw hake | Alcalase | 0.35 | 10.0 | 55 | 5 | Total |
| II-Aut-Blend | Raw hake | Autolysis | -- | 6.8 | 55 | 4 | Partial |
| II-Aut-40* | Raw hake | Autolysis | -- | 6.8 | 55 | 4 | Partial |
| III-A-Blend | Raw alewife | Alcalase | 0.50 | 8.5 | 55 | 5 | Total |
| III-AW-Blend | Raw alewife | Autolysis | -- | 5.0 | 55 | 4 | Partial |
| III-APC-Blend | Alewife Press cake | Alcalase | 0.30 | 8.5 | 55 | 4 | Partial |

* These three products were prepared in 20-gal batches. Each of the other products was a blend, after spray drying, of three 6-liter batches.

are listed in Table 10. Much of the fat was removed from the autolysate by centrifugation before soluble and insoluble solids were blended back together and spray-dried. Cooking and pressing of the alewife in small laboratory equipment were not as effective in oil removal as had been expected. The residual oil in the press cake is reflected in the 25% lipid content of the hydrolysed press cake product. The normal results of industrial pressing operations would yield a product with a much lower fat content and a higher protein concentration.

Table 9.—Average values for proximate and amino acid analyses: replicate runs, three processes, and red hake feed.

| | | | | |
|---|---|---|---|---|
| Enzyme ..... | Pancreatin | Autolysis | Alcalase | -- |
| Concentration ... | 0.5% | -- | 0.35% | -- |
| Type of product .. | Soluble | Whole slurry | Soluble | Raw hake |
| Number of runs .. | 4 | 4 | 3 | 8 |
| Average yield ... | 14.3% | 17.9% | 12.2% | -- |
| Proximate composition (average %): | | | | |
| Protein ..... | 77.23 | 77.85 | 83.71 | 15.89 |
| Moisture ..... | 4.44 | 4.02 | 3.57 | 79.09 |
| Ash ..... | 16.02 | 6.73 | 16.52 | 2.73 |
| Fat ..... | 0.16 | 12.41 | 0.18 | 3.06 |
| Amino acids (% of crude protein): | | | | |
| Lysine ..... | 7.48 | 7.07 | 7.68 | 7.04 |
| Histidine ..... | 1.43 | 1.48 | 1.53 | 1.51 |
| Threonine ..... | 3.84 | 3.53 | 4.02 | 3.60 |
| Valine ..... | 4.38 | 4.53 | 4.67 | 4.50 |
| Methionine .... | 2.66 | 2.73 | 2.81 | 2.69 |
| Cystine ..... | 1.01 | 0.96 | 0.93 | 0.87 |
| Isoleucine ..... | 3.77 | 3.94 | 3.86 | 3.82 |
| Leucine ..... | 6.54 | 6.51 | 7.06 | 6.46 |
| Phenylalanine .. | 3.32 | 3.44 | 3.46 | 3.45 |
| Tyrosine ..... | 2.08 | 2.34 | 1.68 | 2.20 |
| Tryptophan .... | 0.75 | 0.71 | 0.76 | 0.71 |

Table 10.—Average values for proximate and amino acid analyses: replicate runs, three processes, and alewife feed.

| | | | | |
|---|---|---|---|---|
| Enzyme ..... | Alcalase | Autolysis | Alcalase | -- |
| Concentration .... | 0.5% | -- | 0.3% | -- |
| Feed ..... | Raw fish | Raw fish | Press cake | -- |
| Type of product .. | Soluble | Whole slurry | Whole slurry | Raw alewife |
| Number of runs .. | 3 | 3 | 3 | 3 |
| Average yield .... | 13.0% | 18.5% | 18.7% | -- |
| Proximate composition (average %): | | | | |
| Protein ..... | 76.01 | 73.79 | 65.24 | 17.16 |
| Moisture ..... | 4.89 | 4.55 | 4.21 | 71.07 |
| Ash ..... | 16.52 | 5.95 | 9.32 | 2.74 |
| Fat. ..... | 0.21 | 12.56 | 25.07 | 8.35 |
| Amino acids %( of crude protein): | | | | |
| Lysine ..... | 7.97 | 7.33 | 7.42 | 6.77 |
| Histidine ..... | 1.75 | 2.27 | 2.14 | 1.84 |
| Threonine ..... | 3.93 | 3.74 | 3.68 | 3.14 |
| Valine ..... | 4.88 | 4.92 | 4.80 | 4.94 |
| Methionine .... | 2.51 | 2.39 | 2.49 | 2.44 |
| Cystine ..... | 1.13 | 1.07 | 1.22 | 1.26 |
| Isoleucine ..... | 3.99 | 4.08 | 4.14 | 4.09 |
| Leucine ..... | 7.10 | 6.89 | 6.78 | 6.83 |
| Phenylalanine .. | 3.45 | 3.50 | 3.63 | 3.49 |
| Tyrosine ..... | 2.80 | 2.83 | 2.90 | 2.32 |
| Tryptophan .... | 0.88 | 0.65 | 0.61 | 0.79 |

From Tables 9 and 10 it is apparent that the essential amino acids of hydrolysates prepared under these process conditions compare favorably with the corresponding concentrations in the starting material. In the isopropyl alcohol extraction process low quality soluble proteins and nonprotein nitrogen are washed out in the miscella. This results in higher concentrations (as percent of total crude "protein") of essential amino acids in the FPC than are contained in the raw fish.

*Protein efficiency ratios (PER)*—PER were determined by rat feeding trials with the products prepared from hake and alewife. The products prepared from whole red hake were inferior to casein as a sole source of protein in the diets but were statistically equivalent to casein as a supplement to wheat flour. PER for hake products as a sole source of protein are listed in Table 11. The low PER for II-A-40, the 20-gal Alcalese digest, corresponded with a low yield and tryptophan concentration. An excessively high pH was reached during hydrolysis due to an electrode malfunction. The reason for the low PER of II-PC-40 is not clear, but centrifugation was more difficult than for the smaller pancreatin batches and additional heating and acidification were required. Despite the low PER of two of the 20-gal batches as a sole source of protein, they were all very effective as a supplement to wheat flour as is shown in Table 12.

Table 11.—Nutritive quality of hydrolysates produced from red hake by enzymatic hydrolysis.

| Enzyme | Sample No. | Protein efficiency ratio | |
|---|---|---|---|
| | | Actual | % of casein |
| Pancreatin | II-PC-Blend | 2.89 ± 0.07 | 82.2 |
| Pancreatin | II-PC-40 | 2.04 ± 0.13 | 58.0 |
| Alcalase | II-A-Blend | 2.63 ± 0.10 | 74.8 |
| Alcalase | II-A-40 | 2.14 ± 0.07 | 60.9 |
| Autolysis | II-Aut-Blend | 2.87 ± 0.07 | 81.7 |
| Autolysis | II-Aut-40 | 3.13 ± 0.08 | 89.0 |

The results of feeding tests with the alewife (river herring) products are listed in Table 13. From the fatty fish alewife, we finally succeeded in obtaining a totally water-soluble FPC product which was statistically equivalent to casein as a sole source of protein. It was prepared by digestion of the whole fish with the alkaline protease, Alcalase. Partially soluble products prepared from raw whole alewife and from alewife press cake were also equivalent to casein as a sole source of protein.

Several methods were tried for correlation of the PER of various fish protein hydrolysates with the corresponding amino acid analyses. In Figure 9, PER are plotted versus chemical scores based on the amino acid analysis for whole egg as reported by Block and Bolling (1950). The results of earlier feeding trials with hake products prepared with a number of different enzymes and processing conditions are included. Only one of these products was equivalent to casein as a sole source of protein. It was a partially soluble Bromelin digest and included the insoluble sludge which, in this particular case, had been extracted once with isopropyl alcohol. Another product, a soluble pancreatic digest, was supplemented with tryptophan and histidine in a feeding experiment. Neither of the amino acids added alone changed the PER significantly, but when both were added to the same diet there was a marked improvement in PER.

Table 12.—Supplemental value of hake hydrolysates when added to wheat flour (total protein 10% of diet).

| Sample No. | Enzyme | Sample protein - 2% of diet wheat flour protein - 8% | | Sample protein - 4% of diet wheat flour protein - 6% | |
|---|---|---|---|---|---|
| | | PER | Ratio of PER to wheat protein PER | PER | Ratio of PER to wheat protein PER |
| II-PC-40 | Pancreatin | 2.17 ± 0.05* | 1.71 | 2.85 ± 0.05 | 2.24 |
| II-A-40 | Alcalase | 2.09 ± 0.07 | 1.65 | 2.74 ± 0.05 | 2.16 |
| II-Aut-40** | Autolysis | 2.26 ± 0.12 | 1.78 | 2.87 ± 0.08 | 2.26 |
| Casein | -- | 2.28 ± 0.03 | 1.80 | 3.02 ± 0.07 | 2.38 |

[1] PER = Protein efficiency ratio.
* Standard error of the mean.
** Partially soluble product.

Table 13.—Nutritive quality of FPC produced from alewife by enzymatic hydrolysis.

| Protein source | Number of animals | Average daily weight gain | Average daily food intake | Protein efficiency ratio | |
|---|---|---|---|---|---|
| | | | | Actual | % of casein |
| | | *g* | *g* | | |
| Casein ..... | 12 | 5.48 ± 0.30[1] | 14.86 ± 0.50 | 3.54 ± 0.09 | 100.0 |
| III A-Blend[2] ..... | 8 | 4.89 ± 0.25 | 14.24 ± 0.52 | 3.44 ± 0.06 | 97.2 |
| III AW-Blend .... | 12 | 4.61 ± 0.29 | 13.48± 0.65 | 3.34 ± 0.07 | 94.4 |
| III APC-Blend .... | 11 | 4.93 ± 0.24 | 14.24 ± 0.54 | 3.40 ± 0.08 | 96.0 |
| Tukey's W (P<0.05) | -- | 1.02 | 2.10 | 0.28 | |

[1] Standard error of the mean.
[2] See Table 8 for processing details.

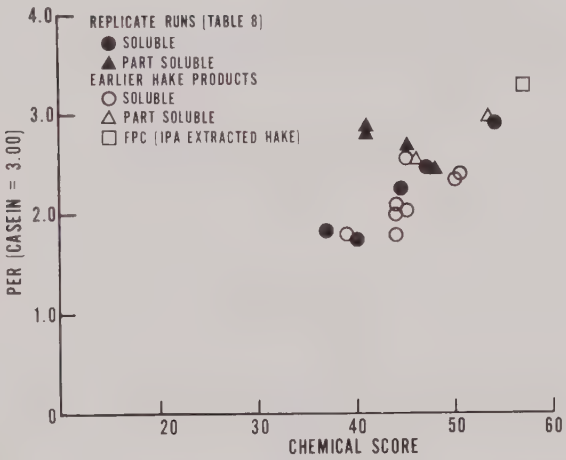


Figure 9.—Protein efficiency ratio (PER) versus chemical score for enzymatic hydrolysates of red hake and alewife.

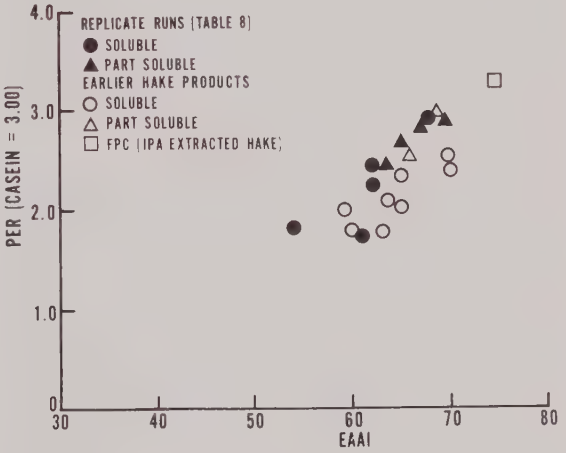


Figure 10.—PER versus Essential Amino Acid Index (EAAI) for fish protein hydrolysates.

The correlation between PER and essential amino acid index (EAAI) is shown in Figure 10. The EAAI is a geometric mean of the essential amino acid concentrations in the sample relative to those in whole egg (limited to 100% for those exceeding whole egg). A good correlation was obtained for the data from the replicate runs, but one product (II-PC-40) and most of the soluble hake products from earlier runs had lower PER for corresponding amino acid indices.

Both chemical score and EAAI are useful as a general guide to the nutritive value of fish protein hydrolysates, but relatively large variations in PER are found between products with similar scores or indices. One probable reason for the variations is that biological availabilities of the amino acids present are not included in the computations. Also, although the amino acid analysis for whole egg as reported by Block and Bolling has generally been used for chemical score calculations, a modified version of it would provide better PER-chemical score correlations.

### Material balances.

*Hydrolysis of red hake.*—Supernatant, sludge, and bone fractions from replicate 6-liter runs were weighed and sampled in order to calculate material balances for each process. Material balances for one run by each of the three processes are summarized in Table 14. Total recoveries for the pancreatin and Alcalese digests are reasonably good. Note the high concentration of lipids in the sludge fractions relative to the supernates. Also note that about one-half of the ash recovered is soluble ash. The autolysate, II-Aut-2, was spray-dried as a whole slurry after removal of bones and scales. Calculated recoveries of lipids and ash are excessive.

Table 14.—Material balances for the processing of red hake by three digestion methods.

| | Enzyme: / Run No.: | Pancreatin II-PC-2 | Alcalase II-A-2 | Autolysis II-Aut-2 |
|---|---|---|---|---|
| Percent recovery protein | Supernate | 72.0 | 65.2 | 90.5 |
| | Sludge | 17.3 | 23.4 | -- |
| | Bones | 6.5 | 6.7 | 8.4 |
| | Total | 95.8 | 95.3 | 98.9 |
| Percent recovery lipids | Supernate | 0.5 | 0.8 | 108.2 |
| | Sludge | 89.2 | 83.4 | -- |
| | Bones | 5.8 | 3.2 | 4.4 |
| | Total | 95.5 | 87.4 | 112.6 |
| Percent recovery ash[1] | Supernate | 42.4 | 43.6 | 48.2 |
| | Sludge | 17.5 | 23.4 | -- |
| | Bones | 35.9 | 25.3 | 70.1 |
| | Total | 95.8 | 92.3 | 118.3 |

[1] Based on ash content of fish plus ash added during pH adjustment and control.

A flow diagram with more detailed material balance information for preparation of a totally soluble hydrolysate using pancreatin, Run No. II-PC-2, is shown in Figure 11.

*Hydrolysis of alewife.*—Material balances for one run by each of the three processes employed with alewife are summarized in Table 15. Dry solids recoveries of about 80% were obtained when the various supernates were spray-dried. Recoveries nearer to 95% would be expected in a continuous operation with a secondary bag collector. Therefore, product yields calculated were based on dry solids contents of the supernates.

More detailed material balance information is shown for runs yielding a soluble product from raw alewife (Figure 12) and a partially soluble product from press cake (Figure 13).

## SUGGESTED PROCESS OUTLINES

### Raw Fish

**Fatty fish.**—The processing of raw, fatty fish for the production of a dry soluble concentrate and/or fish paste is outlined in Figure 14. Raw fish are chosen because the active autolytic enzymes are required in combination with an added commercial proteolytic enzyme in order to obtain economically the desired degree of solubilization. Water equal in weight to the fish is metered in during comminution.

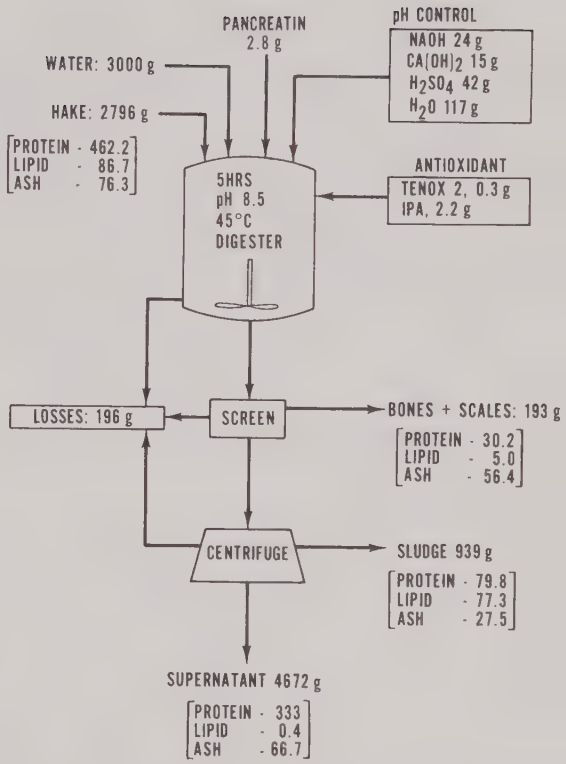


Figure 11.—Material balance, Run No. II-PC-2: totally soluble product from red hake.

Table 15.—Material balances for the processing of alewife (river herring) by three digestion methods.

| | Enzyme: / Run No.: | Alcalase III-A-3 | Autolysis III-AW-3 | Alcalase[1] III-APC-3 |
|---|---|---|---|---|
| Percent recovery protein | Supernate | 61.2 | 86.0 | 78.2 |
| | Sludge | 31.9 | [2] 4.4 | -- |
| | Bones | 5.2 | 9.6 | 8.1 |
| | Total | 98.3 | 100.0 | 86.3 |
| Percent recovery lipids | Supernate | 0.3 | 20.1 | 58.3 |
| | Sludge | 88.6 | [3]53.3 | [3]30.7 |
| | Bones | 1.7 | 3.6 | 3.9 |
| | Total | 90.6 | 77.0 | 92.9 |
| Percent recovery ash[4] | Supernate | 40.2 | 34.8 | 61.1 |
| | Sludge | 18.0 | [3] 2.6 | -- |
| | Bones | 36.0 | 47.8 | 51.4 |
| | Total | 94.2 | 81.2 | 112.5 |

[1] Digestion of press cake; recoveries based on weight of fish to cooker.
[2] Fat and oil fraction removed by centrifugation.
[3] Free oil separated by pressing of cooked fish.
[4] Based on ash content of fish plus ash added during pH adjustment and control.

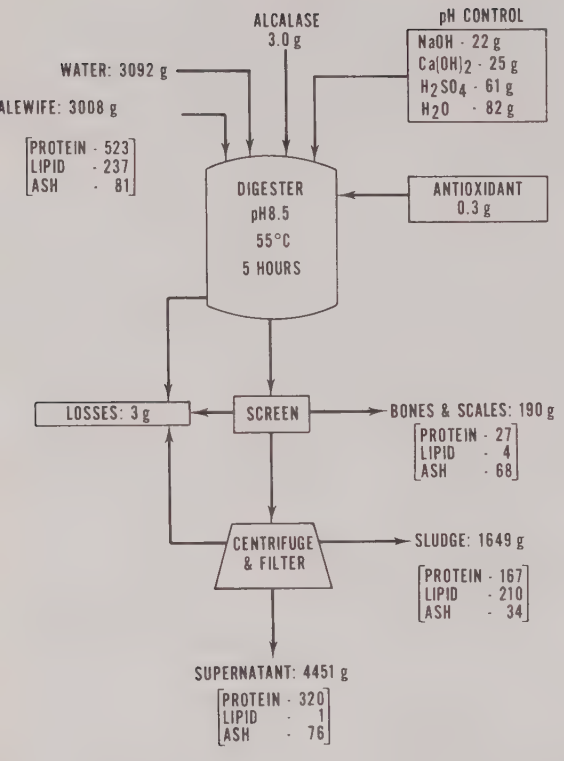


Figure 12.—Material balance, Run No. III-A-3: soluble product from alewife.

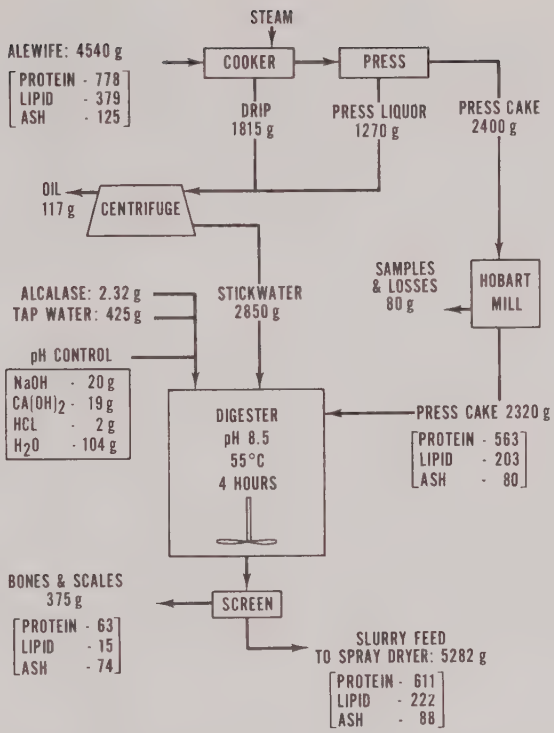


Figure 13.—Material balance, Run No. III-APC-3: partially soluble product from alewife press cake.

*Enzymatic hydrolysis.*—The fish slurry is hydrolysed in an agitated jacketed reaction vessel. A minimum of three reaction vessels are used in the plant design so that a constant feed of hydrolysate to subsequent processing equipment may be maintained. The enzyme of choice is an alkaline protease of *Bacillus subtilis* added at a level of 0.1 lb. per 100 lb. of fish. Commercial preparations such as Alcalase of Novo Industries, P-58 of Rohm and Haas or Enzeco ABP of Enzyme Development Corporation may be used. An antioxidant is also added to the digestion mixture or is added with the extra water during fish comminution. Propyl gallate at a level of 1.4 g (0.003 lb.) per 100 lb. of fish is recommended.

The fish slurry is adjusted to about pH 8.5 by adding $Ca(OH)_2$ at the rate of 0.85 lb. per 100 lb. of fish. The pH is maintained at 8.5 during hydrolysis by the addition of a 5N solution of NaOH via an automatic pH controller. The temperature is automatically controlled at 55°C during the 5-hr hydrolysis period. The hydrolysed slurry is acidified to pH 6.5 with $H_2SO_4$ as it flows into the centrifuge hold tank.

*Bone feed product.*—Bones and scales are separated from the hydrolysis mixture by a vibrating screen as it leaves the reactor. Suspended undigested solids and fine bone particles are then removed by the disc-type centrifuge. The sludge discharge from the centrifuge is combined with the wet bone fraction and conveyed to a rotary steam tube dryer. The dried mixture (10% moisture) is weighed, bagged, and stored as bone meal.

*Fish oil.*—The oil discharge stream from the centrifuge is washed with hot water as it flows into a hold tank. The free oil is then separated in a liquid-liquid centrifuge and is later pumped from the collection tank to the fish oil storage tanks.

*Soluble concentrate.*—The aqueous main stream discharge from the centrifuge is collected in a hold tank and then concentrated to a dry solids content of about 50% in a wiped-film evaporator. The concentrate is dried in a spray dryer with a secondary bag collector, weighed, bagged, and stored as dry FPC. As an alternative the concentrate from the evaporator may be packaged in 5-gal cans and stored for sale as fish paste.

**Lean fish.**—Hydrolysates have been prepared from the lean fish, red hake, using a wide variety of enzymes and processing conditions, but we have not yet succeeded in obtaining a totally soluble product from hake which is nutritionally equal to casein as a sole source of protein. The recommended process for lean fish, therefore, is one in which a partially soluble product is prepared. A satisfactory product with good nutritive value has been prepared from hake by autolysis without added enzymes for a 4-hr period at 55°C. The hydrolysis period can be reduced to less than 3 hr by the addition of a low concentration (e.g., 0.02 lb/100 lb of fish) of an enzyme such as ficin. Digestion at neutral to slightly acid conditions does not require any pH adjustment.

Referring to Figure 14, the bones and scales are screened out and dried for bone meal, but the sludge discharge from the centrifuge is recombined with the aqueous stream prior to evaporation and spray drying. The partially soluble product can be produced quite cheaply since little or no extra enzyme is added, and the yield of product is higher, naturally, when insoluble solids are included. The product is darker in color than the isolated soluble fraction, however, and has a much higher residual fat content.

## Press Cake (Fatty Fish)

A fish protein concentrate with good nutritive value can be produced cheaply through the use

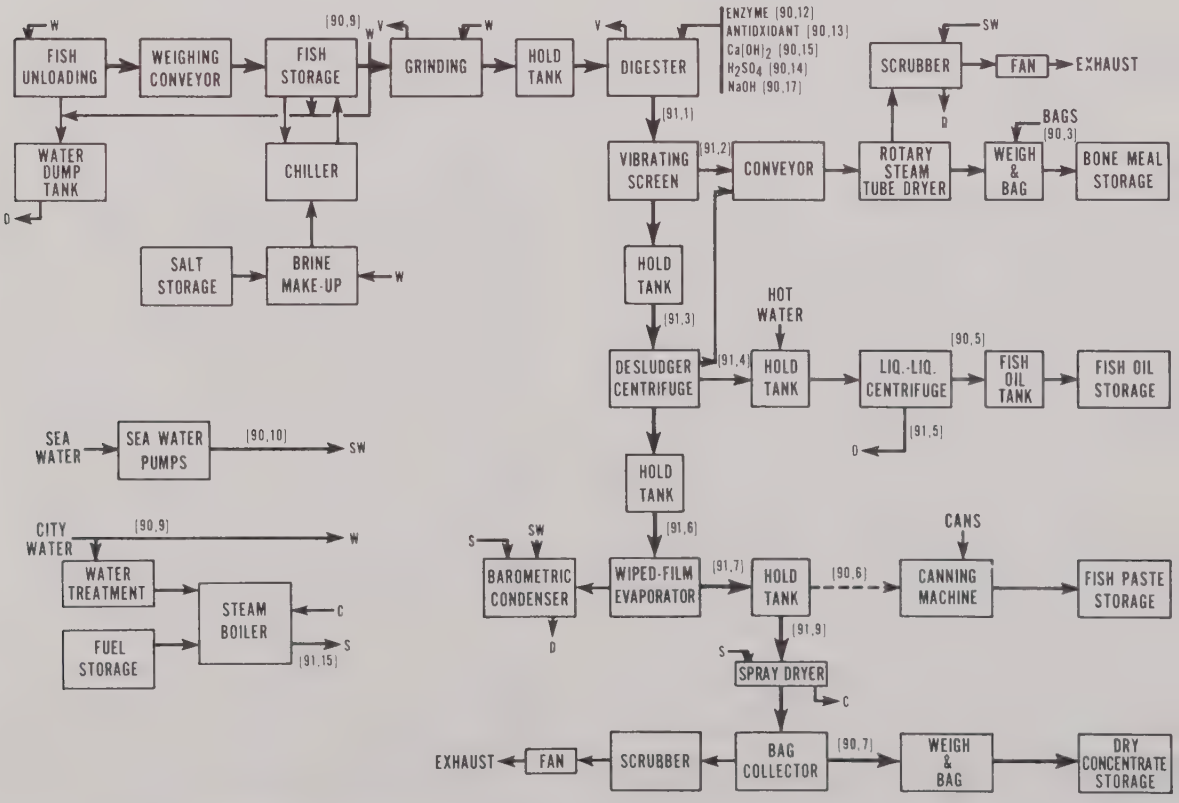


Figure 14.—Suggested process outline for soluble product from raw fish by enzymatic hydrolysis.

of enzymes to partially solubilize the cake produced by the pressing of fatty fish. A totally soluble FPC from press cake would probably not be feasible because the native fish enzymes are inactivated in the initial cooking step. An uneconomical level of commercial enzyme would be required to obtain a good yield of soluble product with an adequate amino acid profile.

**Feed to digesters.**—A process outline based on press cake is shown in Figure 15. The press cake feed is prepared by the standard wet reduction process of the fish meal industry. The whole fish are cooked and then pressed in a continuous screw press. The press liquor is screened to remove suspended solid "foots" and then fed from a hold tank to a centrifuge where the crude fish oil is removed. The oil is clarified by a hot water wash and a second centrifugation before it is pumped to oil storage. The stickwater from the first centrifugation is fed to the digesters and mixed with the press cake to form the slurry for hydrolysis. The system of evaporators used in a fish meal plant to prepare concentrated solubles from stickwater is not required.

**Enzymatic hydrolysis.**—A product with PER equal to that of casein has been prepared using the alkaline protease, Alcalase, at a level of 0.1 lb. per 100 lb. of alewife press cake. Bromelin or ficin might be more suitable since no pH adjustment would be required. After a 3-4 hr hydrolysis at 55°-60°C, the bones are separated from the hydrolysate on passage through a vibrating screen.

**Processing.**—Additional oil may be removed from the hydrolysate by centrifugation, but experimental results thus far indicate that the degree of oil removal possible by this means does not justify the inclusion of a centrifuge at this stage of the process. The lipid content of the FPC would then depend on the efficiency of the pressing operation. Based on normal fish meal operations a level of 6 to 10% residual lipids on a dry weight basis would be expected. This level could be reduced by hot water washing of the press cake prior to hydrolysis. If most of the solids could be recovered by screening, the wash water would be mixed with the press liquor for centrifugation. After oil separation the

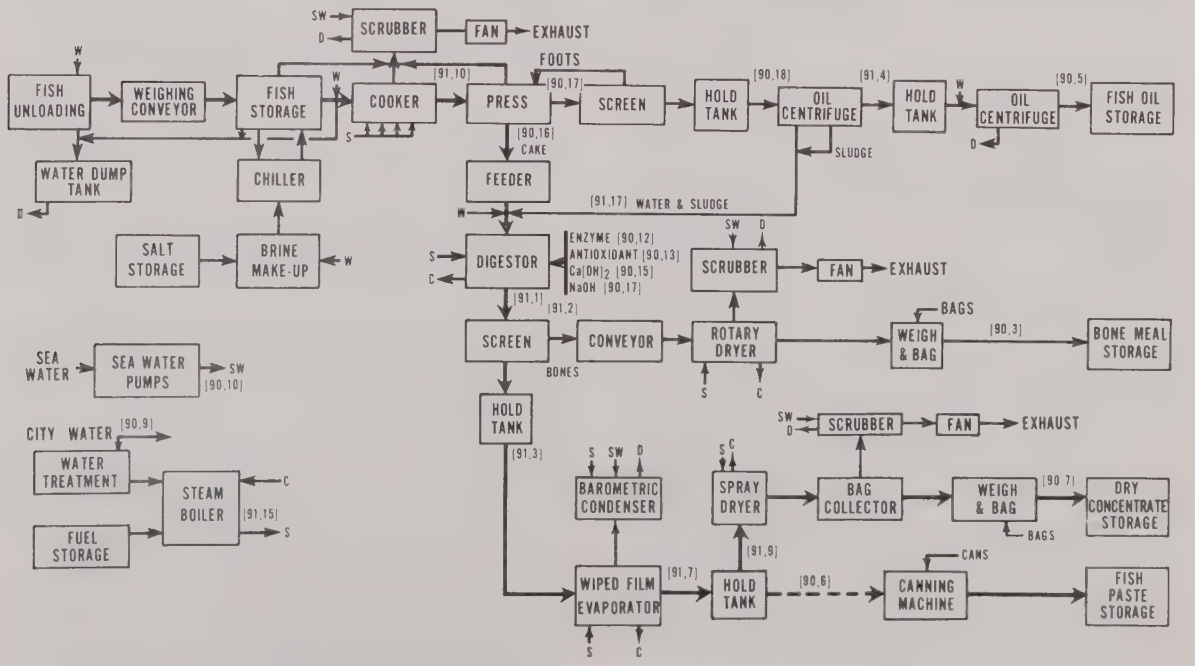


Figure 15.—Suggested process outline for production of an FPC from fatty fish press cake by enzymatic hydrolysis.

suspended solids would be returned to the reactor vessel with the stickwater.

The bones, which are removed by screening, are dried, packaged, and stored as bone meal. The hydrolysate may be passed directly to the wiped-film evaporator feed tank after screening. If the hydrolysate is centrifuged for additional oil removal, the insoluble solids are recombined with the aqueous stream, concentrated by evaporation and either spray-dried or canned as fish paste.

## Cost Estimates

A computer program entitled "Engineering Economic Model for Fish Protein Concentrate Processes" has recently been prepared under a Bureau of Commercial Fisheries contract with the Chemical Engineering Department of the University of Maryland. It estimates plant construction and operating costs for various types of FPC processes and has been used here in the estimation of production costs for hydrolysates of raw fish and fish press cake.

Input data to the computer include: plant size; operating days per year; costs of fish, utilities, and labor; depreciation and interest rates; proximate composition of fish; and a current cost index. Enzyme cost and effectiveness are also included for the biological processes.

From process flow sheets and distribution equations, preferably based on experimental results, material and energy balances are computed for each process. Major pieces of process equipment are then sized and tabulated along with estimated installed costs. Costs of specific pieces of process equipment, facilities, fish handling systems, etc. provide a consistent basis for comparison between alternate FPC processes.

**Input data.**—Cost estimates were based on the assumption of fish costing 1 cent per pound and composed of 12% oil, 16% protein, and 3% ash. Electricity at 1.3¢/KWH, fuel at 6¢/therm, and labor at $4/hr were also assumed. The charge for depreciation plus interest was set at 15% per annum. Enzyme, costing $5/lb, was specified at 0.1 lb. per 100 lb. of raw fish (soluble product) and at 0.1 lb. per 100 lb. of press cake (partially soluble product).

**Summary of results.**—Equipment for each process was sized and tabulated with estimated installed cost on the computer print-outs. Estimated fixed capital costs for 200 ton/day plants are listed in Table 16. A depreciation and interest charge of 15% per year on fixed capital costs was included in computations of total annual operating costs for each process.

Annual operating costs and production rates computed by the Engineering Economic Model were used to calculate production costs for dry concentrates of fish protein. These cost estimations for both 50 and 200 ton/day plants are summarized in Table 17. By-product values of $80 per ton of crude fish oil and $60 per ton of bone feed were assumed for the computations.

The estimated production cost for a soluble FPC prepared by the biological process is a little higher than that estimated for FPC prepared by isopropanol (IPA) extraction of whole fish. It is not as expensive, however, as one prepared by IPA extraction of wet deboned hake. The straight biological process has the advantage of a lower capital cost than any of the other FPC processes studied.

Based on computer estimates the press cake biological process yields a product that is significantly cheaper than the IPA-extracted FPC. If the residual oil can be suitably stabilized the press cake product will add extra caloric value as well as excellent protein to deficient diets.

The assumed price of 1 cent per pound for fish may not be valid, particularly for lean fish. If the fish price were doubled to 2¢/lb, the costs

Table 16.—Estimated fixed costs for the construction of biological and press cake biological FPC plants.

| | Process | |
|---|---|---|
| | Biological | Press cake biological |
| Capacity, tons of fish per day .... | 200 | 200 |
| Feed to reactor ..... | Raw fish (fatty fish) | Press cake (fatty fish) |
| Fixed costs: | | |
| Equipment ..... | $1,783,257 | $1,985,130 |
| Spare parts ..... | 20,906 | 26,250 |
| Facilities ..... | 210,348 | 210,348 |
| Engineering ..... | 151,088 | 166,630 |
| Contingencies .... | 216,560 | 238,836 |
| Total capital costs | $2,382,159 | $2,627,194 |

Table 17.—Cost estimates for biological and press cake (fatty fish) biological processes, 200 operating days per year.

| | Process | | | |
|---|---|---|---|---|
| | Biological | | Press cake biological | |
| Capacity, tons/day | 50 | 200 | 50 | 200 |
| Total capital costs | $1,282,492 | $2,382,159 | $1,316,279 | $2,627,194 |
| Oil, tons/year | 900 | 3600 | 953 | 3811 |
| Value @ $80/ton | $72,000 | $288,000 | $76,240 | $304,880 |
| Bone feed, tons/year | 1338 | 5353 | 345 | 1380 |
| Value, @ $60/ton | $80,280 | $321,180 | $20,700 | $82,800 |
| Total by-product value, $/year | $152,280 | $609,180 | $96,940 | $387,680 |
| Total operating cost, $/year | $971,097 | $2,753,790 | $877,614 | $2,400,400 |
| Net cost, $/year | $818,817 | $2,144,610 | $780,674 | $2,012,720 |
| Dry concentrate, tons/year | 1364 | 5455 | 1965 | 7858 |
| Assigned cost, $/ton | $600 | $394 | $397 | $256 |
| Est. production cost, cents/lb | 30.0¢ | 19.7¢ | 19.9¢ | 12.8¢ |

for enzymatic products would be increased about 25% at 50 tons/day and about 40% at a 200 tons/day plant capacity. (The same increase in cost per pound represents a smaller percentage of the higher cost at 50 tons/day.)

The production costs listed in Table 17 include amortization of capital costs but do not include marketing costs and profit margins. Selling prices might be in the range of 30 to 50% higher.

## PRODUCT CHARACTERISTICS AND USES

### Characteristics

**Soluble product.**—Totally soluble products, prepared by spray drying of clarified hydrolysates, are fine powders of low bulk density. They are more or less hygroscopic and should be stored in sealed containers.

Soluble FPC prepared from hake are off-white to light yellow in color. Odors are mildly cheeselike and in some products somewhat fishy. Taste is moderately salty and cheeselike with a slightly fishy aftertaste. The soluble product prepared from alewife was light yellow in color and had a malty, slightly fishy odor. Taste was somewhat fishy with a slightly bitter aftertaste.

A typical soluble FPC prepared at pH 8.5 will contain 78 to 80% crude protein, about 0.5% lipids, about 16% soluble ash, and less than 5% moisture. Based on chemical scores, isoleucine is usually the limiting amino acid for products prepared with alkaline proteases. Methionine is next limiting for alewife products and tryptophan for hake products.

**Partially soluble product.**—Products that are 50 to 70% water-soluble have been prepared by autolysis of raw fish and also by enzymatic hydrolysis of press cake. An FPC prepared from alewife press cake had an orange-brown color and a sweet, fish meal odor. Although it had a good protein efficiency ratio, it had a very high lipid content and a definitely fishy taste. A spray-dried hake autolysate was a gray powder containing 12% lipids and had a less fishy taste and odor.

The amino acid patterns of the partially soluble products were in general similar to those of the raw fish. Protein efficiency ratios for all of the products were essentially equivalent to that of casein.

### Uses

Totally soluble protein hydrolysates of whole fish can be used in food products such as soups and beverages where solubility is desirable if a bland flavor is not required. Nutritionally, they are particularly effective as a wheat supplement. Aside from human food items they might also find use as a milk replacer in calf feeding ration, etc., where a cheap product with low fat content is needed. Changes in processing conditions or raw material may be required to produce a blander soluble product suitable for human beverage formulations.

A partially soluble fish hydrolysate product is readily dispersable. It has a higher lipid content but is inexpensive. It is physically stable but has a moderate fish meal odor which may intensify during storage. It has good nutritive value both as a supplement and as a sole source of protein and might be used as a protein supplement in cultures or locales where its taste would be acceptable and the caloric value of the lipid desirable.

Fish protein hydrolysates are probably most suited for use in dry soup mixes, broths, or bouillons. With flavor modifications they might satisfy the need for a soluble protein concentrate to fortify beverages. They could very well be included in savory crackers, sandwich spreads, and pastes. The flavor and nutritive value of certain overly bland processed meats and meat extenders might be improved by inclusion of the fish hydrolysate.

Whether or not the enzymatically produced FPC is suitable for inclusion in bread has not yet been determined but bread fortified with it would have good nutritional value. Soluble FPC would not be suitable for direct incorporation into pasta products because they would be leached out in cooking. They might, however, be used in sauce formulations for pasta.

Limited food research has been carried out with the hydrolysate products prepared in the replicate runs. They were incorporated at a level of 10% in plain sugar cookies which were evaluated for flavor, texture, and appearance. Control cookies containing no FPC and cookies containing IPA-extracted hake FPC were included for comparison. The cookies containing fish hydrolysates were judged to be significantly poorer in flavor. Texture was also different with the hydrolysate-containing cookies being somewhat brittle rather than crumbly. The test cookies were thinner than the controls, but they were not statistically different in appearance. Although the general flavor characteristics of fish protein hydrolysates would be more acceptable in types of food other than cookies, the flavor poses an important problem to be solved. Additional research is needed on the preparation, processing, and utilization in various foods of biological FPC before they can be considered acceptable for use in most American-type foods.

## STATUS AND FUTURE OF BIOLOGICAL FPC METHODS

Many different approaches to the preparation of FPC by biological procedures have been tried by various investigators. A completely satisfactory commercial process having human food applications has not as yet been demonstrated. Fish can be preserved inexpensively in a concentrated form through biological procedures, but a bland product of the type that may be prepared by solvent extraction is difficult if not impossible to prepare by biological procedures alone. Some proposed processes combine solvent extraction with enzymatic or microbial treatment but complete elimination of the use of solvents would be desirable and a truly bland product from such a combination is unlikely.

A water-soluble FPC is desirable for certain applications and this can be prepared by using proteolytic enzymes. The nutritive value of soluble FPC may be limited by inadequate concentrations of certain essential amino acids unless suitable processing conditions are chosen. We have found that hydrolysis above pH 8.5 with alkaline proteases of *Bacillus subtilis* gives good yields and amino acid patterns for soluble products. The species of fish processed is also a factor in the level of nutritive quality which can be reached.

The physical, chemical, and organoleptic characteristics of enzymatically prepared FPC have not been fully determined and described in this report because additional product development is needed. Organoleptic characteristics, particularly flavor, are the properties of biologically prepared FPC which presently limit their applications. For this reason food research with the products has also been quite limited.

The first goal of additional research with soluble hydrolysates of whole fish should be improvement of organoleptic and physical characteristics while retaining good nutritive value. Additional engineering studies in areas such as centrifugation and mixing power requirements would then be appropriate prior to final scale-up.

One possible approach to the primary goal of flavor modification is fermentation of enzymatic hydrolysates with suitable microorganisms. Yeast (Jeffreys and Krell, 1965) and carbohydrate fermenting bacteria (Tada and Nakayama, 1956; Krishnaswamy et al., 1965) have been

used, with small amounts of added carbohydrate, to modify fish flavor and odor.

The use of ultrafiltration in the processing of soluble hydrolysates is another interesting approach which might improve product characteristics. Rapid advances are being made in membrane technology and ultrafiltration membranes are probably available which could remove excess salt and possibly some undesirable nonprotein nitrogen while retaining peptides and most free amino acids. The concentration of the hydrolysate affected by ultrafiltration would also reduce the evaporator or dryer requirements.

The hygroscopic nature and low bulk density of the spray-dried soluble powders are physical properties which could stand improvement. These problems would be avoided, however, if the hydrolysates were marketed as concentrated pastes.

The partially soluble FPC that was prepared from alewife press cake and described in this report had very poor organoleptic characteristics. These characteristics can undoubtedly be improved by certain changes in the process and operating techniques without significantly affecting the low cost and good nutritive value of the product. A truly bland product probably will not be obtained, however, without the additional use of chemical solvents.

There is presently a demand for a soluble protein concentrate which could be used in carbonated beverages. No product has yet been developed which meets all the requirements. A product that is soluble, nutritious, and relatively bland in taste would command a premium price. Considerable improvement in the flavor of soluble hydrolysates of whole fish would be required for this application. Enzymatic solubilization of a solvent extracted FPC may be the means for filling this need if the price is right. Steam stripping of the FPC would be neither necessary nor desirable.

There are certain nonfood uses to which enzymatically prepared FPC might be applied in the near future. The financial risks of constructing an FPC plant are reduced if the plant can be used primarily for animal feed production until an FPC market is developed. The use of soluble FPC as a partial milk replacement for calf feeding should be investigated. Another possible use is in microbiological culture media. Significant amounts of casein hydrolysates, relatively high priced, are presently used for this purpose.

Biological procedures would offer advantages over a solvent extraction process for either shipboard processing or small-scale village industry operations. The problems of solvent transportation, storage, and recovery would be eliminated. On board ship the freshly caught fish could be comminuted and by proper control of pH and temperature be preserved while undergoing partial hydrolysis. Pumping of the slurry to tanks on shore for final processing would be easy. A simple biological process with minimal equipment costs and suitable controls to insure product safety would be appropriate for village industry operations.

In summary, a variety of biological procedures are available which offer interesting alternatives to FPC production by solvent extraction. Advantages in terms of functional properties and/or costs of FPC products are possible in many cases. A process has been developed for the preparation of a totally water-soluble FPC with good nutritive value through the enzymatic hydrolysis of whole fish. A partially soluble FPC prepared from fish press cake has also been described and preliminary cost estimates have been made for both processes. The principal factor limiting food applications of these and other biologically prepared products are their taste characteristics. Flavor improvement should be the primary goal of additional research and development.

## SUMMARY

Fish protein concentrates (FPC) may be prepared by various biological, as well as chemical, procedures. Both microbial cultures and isolated enzymes have been used for this purpose. This report summarizes research that has been carried out by the National Marine Fisheries Service (formerly Bureau of Commercial Fisheries) on biological methods for FPC production. The effects of various commercially available proteolytic enzymes and processing conditions on yields and characteristics of water-soluble fish protein hydrolysates is presented. Soluble products tended to be deficient in either tryptophan or histidine depending on the pH of hydrolysis. Hydrolysis of raw fish with an alkaline protease of *Bacillus subtilis* at pH 8.5 or

above gave the best balance of essential amino acids. The protein efficiency ratio (PER) of a totally soluble product prepared from alewife was equivalent to that of casein. Soluble FPC prepared from red hake were equivalent to casein as a wheat supplement but not as a sole source of protein. Possible food uses and the need for improvement of flavor characteristics in the fish protein hydrolysates are discussed. Process outlines and cost estimates are presented for the production of (1) a totally soluble product using the alkaline protease and (2) a partially soluble product prepared by the hydrolysis of press cake. A literature survey of fish protein modifications by related biological and hydrolysis methods is included in the report.

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# APPENDIX

Suppliers and classifications of enzymes tested.

| Manufacturer and/or supplier | Enzyme classification by source | | |
|---|---|---|---|
| | Plant | Animal | Microbial |
| Calbiochem<br>Los Angeles, Calif. | -- | -- | Pronase |
| Cudahy Laboratories<br>Omaha, Neb. | -- | Pepsin<br>Pancreatin | -- |
| Enzyme Development Corp. (EDC)<br>New York, N.Y. | Ficin<br>Papain | -- | Alcalase[1]<br>Enzeco Alk. Bact. Prot.<br>Bact. Protease Novo |
| Miles Laboratories<br>Elkhart, Ind. | Bromelin<br>Ficin<br>Papain | -- | Fungal Protease<br>HT Proteolytic |
| Monsanto Company<br>St. Louis, Mo. | -- | -- | Monzyme PA-1 |
| Nutritional Biochem. Corp. (NBC)<br>Cleveland, Ohio | Bromelin<br>Ficin<br>Papain | Pepsin<br>Trypsin | -- |
| Rohm and Haas Co.<br>Philadelphia, Pa. | -- | -- | Rhozymes P-11 & 41<br>Rhozyme P-F Conc.<br>E56 & P58 Alkaline |
| Wilson Laboratories<br>Chicago, Ill. | -- | Pepsin<br>Trypsin | -- |
| Wallerstein Company<br>Staten Island, N.Y. | Papain | -- | Prolase MT-7820 |

[1] Microbial proteases manufactured by Novo Industries, Copenhagen.

GPO 985-371

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23. Apparent abundance, distribution, and migrations of albacore, *Thunnus alalunga*, on the North Pacific longline grounds. By Brian J. Rothschild and Marian Y. Y. Yong. September 1970, v + 37 pp., 19 figs., 5 tables.

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28. Fur Seal Investigations, 1969. By NMFS, Marine Mammal Biological Laboratory. August 1971, 82 pp., 20 figs., 44 tables, 23 appendix A tables, 10 appendix B tables.

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30. Blue crab meat. I. Preservation by freezing. July 1971, iii + 13 pp., 5 figs., 2 tables. II. Effect of chemical treatments on acceptability. By Jurgen H. Strasser, Jean S. Lennon, and Frederick J. King. July 1971, iii + 12 pp., 1 fig., 9 tables.

31. Occurrence of thiaminase in some common aquatic animals of the United States and Canada. By R. A. Greig and R. H. Gnaedinger. July 1971, iii + 7 pp., 2 tables.

32. An annotated bibliography of attempts to rear the larvae of marine fishes in the laboratory. By Robert C. May. August 1971, iii + 24 pp., 1 appendix I table, 1 appendix II table. For sale by the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402 - 35 cents.

33. Blueing of processed crab meat. II. Identification of some factors involved in the blue discoloration of canned crab meat *Callinectes sapidus*. By Melvin E. Waters. May 1971, iii + 7 pp., 1 fig., 3 tables.

634. Age composition, weight, length, and sex of herring, *Clupea pallasii*, used for reduction in Alaska, 1929-66. By Gerald M. Reid. July 1971, iii + 25 pp., 4 figs., 18 tables.

635. A bibliography of the blackfin tuna, *Thunnus atlanticus* (Lesson). By Grant L. Beardsley and David C. Simmons. August 1971, 10 pp. For sale by the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402 - 25 cents.

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